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㉘ **Novel enzyme product and its use in the saccharification of starch.**

㉙ A novel acid amylase is provided, isolated from amyloglucosidase, having a greater thermostability than amyloglucosidase and a comparable pH-optimum. This acid amylase can be used advantageously in conjunction with amyloglucosidase and optionally with an acid pullulanase in the saccharification of starch and starch hydrolysates. Also, a novel enzyme product is provided comprising acid amylase having  $\alpha$ -1, 4-glucosidic bond splitting activity and amyloglucosidase and optionally acid pullulanase.

NOVEL ENZYME PRODUCT AND ITS USE IN THE SACCHARIFICATION OF STARCH

FIELD OF THE INVENTION

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The present invention relates to enzymatic starch degradation. More specifically, the invention provides a novel enzyme product useful in the saccharification of starch, especially liquefied starch, and a process for its preparation.

STATE OF THE ART

Native starch is known to contain two types of macromolecules composed of glucose units. One type of molecule, called amylose, is linear and consists exclusively of  $\alpha$ -1,4-linked glucose units. Starch contains about 25% of amylose. The second type of molecule, called amylopectin, is highly branched and contains  $\alpha$ -1,4 as well as  $\alpha$ -1,6 linked glucose units. The overall content of  $\alpha$ -1,6 linkages is generally less than 5%.

Sugars from starch, in the form of concentrated dextrose syrups, are currently produced at the rate of several million tons per annum by a two stage enzyme catalyzed process involving: (1) liquefaction (or thinning) of solid starch with an  $\alpha$ -amylase into dextrans having an average degree of polymerization of about 7-10, and (2) saccharification of the resulting liquefied starch (i.e. starch hydrolysate) with amyloglucosidase, which results in a syrup of high glucose content (92-96% by weight of the total solids). Much of the dextrose syrup produced commercially is then enzymatically isomerized to a dextrose/fructose mixture known as isosyrup.

The two enzymes used,  $\alpha$ -amylase and amyloglucosidase, differ in two important aspects. First,  $\alpha$ -amylase, which is a so-called endo-enzyme, attacks macromolecules at random. Amyloglucosidase, on the other hand, is a so-called exo-enzyme and splits glucose units successively from the non-reducing end of the dextrin molecule in the

starch hydrolysate. Secondly,  $\alpha$ -amylase exclusively attacks  $\alpha$ -1,4 linkages whereas amyloglucosidase splits  $\alpha$ -1,6 linkages as well.

The recommended name of amyloglucosidase is  $\alpha$ -1,4-D-glucosidase, the Enzyme Committee number 3.2.1.3 and the systemic name  $\alpha$ -1,4-glucan glucohydrolase. Amyloglucosidase is also called AG or glucoamylase and it will be understood that the terms amyloglucosidase, AG and glucoamylase, as used hereinafter, are synonymous.

10       Whereas amylopectin is only partially degraded by  $\alpha$ -amylase because this enzyme exclusively attacks  $\alpha$ -1,4 linkages, substantial hydrolysis of the branched oligosaccharides occurs in the subsequent saccharification step catalyzed by amyloglucosidase which also hydrolyses  $\alpha$ -1,6  
15 glucosidic linkages, though at a considerably lower rate than the  $\alpha$ -1,4 linkages.

The saccharification stage of the commercial process outlined above has long been recognized to be deficient in certain regards. In particular, the amyloglucosidases  
20 currently available catalyse both saccharification and dextrose reversion reactions, e.g. conversion of dextrose into isomaltose, at rates which depend on the substrate concentration. The formation of by-products in this way has limited the saccharification of starch hydrolysates into  
25 dextrose to not more than about 95% by weight of dextrose on dry solids basis (hereinafter termed DX) in syrups containing at least 33% dry solids by weight.

It is true that the formation of by-products from reversion reactions may be suppressed by up to about 50% with  
30 a concomitant increase of starch conversion of about 1-2% if a relatively high level of amyloglucosidase combined with a dilution of the substrate to about 15% dry solids is employed (cf. U.S. Patent No. 4,017,363), but the concentration of the resulting dextrose solution to the conventional higher dry  
35 solids levels is energy consuming.

In an effort to further increase the DX value it has been proposed to use a debranching enzyme, in conjunction with amyloglucosidase, so as to hydrolyze more efficiently the branched oligosaccharides (containing  $\alpha$ -1,6 glucosidic bonds)

present in the liquefied starch.

European Patent Application No. 82302001.1, Publication No. 0 063 909, describes a debranching enzyme of the pullulanase type which is produced by a Bacillus called  
5 Bacillus acidopullulyticus. According to this specification the debranching enzyme has optimum activity at a pH in the range of 3.5 to 5.5 (under defined conditions) and its thermal activity optimum at pH 4-5 is at least about 60°C. The residual activity after 72 hours at 60°C at pH 5 is 50% or  
10 more. This acid pullulanase is used together with one of the saccharifying enzymes amyloglucosidase or  $\beta$ -amylase. The use of this acid pullulanase in conjunction with amyloglucosidase is reported to result into a higher dextrose level which is higher by about 1% as compared with the level obtained with  
15 amyloglucosidase alone under similar conditions. Alternatively the same dextrose level may be achieved using about half the amount of amyloglucosidase.

U.S. Patent No. 4,335,208 discloses the combined action of amyloglucosidase and another debranching enzyme,  
20 namely isoamylase from Pseudomonas amyloclavata. According to this reference the isoamylase has a pH optimum close to that of amyloglucosidase so that the amount of the latter can be considerably reduced to obtain the same or even a higher dextrose level than with amyloglucosidase alone. However, the  
25 process has a serious drawback in that the isoamylase is heat labile. This means that no saccharification in the presence of isoamylase is technically feasible above about 55°C, whereas amyloglucosidase by itself is normally used at 60°C in the saccharification of starch hydrolysate. Moreover, micro-  
30 organisms of the genus Pseudomonas are not so-called GRAS-microorganisms (Generally Regarded As Safe), so that enzymes produced by such microorganisms are not permitted in food and food processing in the USA.

U.S. Patent No. 3,897,305 discloses the combined use  
35 of amyloglucosidase and pullulanase from Aerobacter aerogenes (Klebsiella pneumoniae) which is stated to give an increase in DX of up to 2% in syrups containing at least 30% dry solids. Practically no saving of amyloglucosidase is achieved however, because of the unfavourable pH optimum (5.5-6.0), of the

enzyme from K. pneumoniae which makes it necessary to conduct the saccharification at a relatively high pH at which the activity of amyloglucosidase is severely reduced.

Marshall et al (Febs Letters, Vol. 9 No. 2, July 5 1970, pages 85-88) reported that amyloglucosidase obtained from Aspergillus niger contained an  $\alpha$ -amylase-like impurity apparently essential for complete hydrolysis of starch to glucose. No attempt was however made to characterize or isolate this impurity.

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#### OBJECTS OF THE INVENTION

It is an object of the invention to provide a novel acid amylase, which can be derived from amyloglucosidase 15 preparations, having substantial  $\alpha$ -1,4-glucosidic bond splitting activity. The novel enzyme product has  $\alpha$ -glucosidic bond splitting activity at acidic pH, and can be used in the saccharification of starch and, preferably, liquefied starch.

It is a further object of the invention to provide a 20 novel process for converting starch into syrups with a high dextrose content.

#### THE INVENTION

25 According to its first aspect the present invention provides a microbial acid amylase, obtainable from amyloglucosidase, and having substantial  $\alpha$ -1,4-glucosidic bond splitting activity. This acid amylase effects optimum saccharification at a pH between 3.5 and 5.0 at temperatures 30 from about 60 to about 75°C. Under ordinary storage conditions it is stable over a period of several months.

The acid amylase of the invention occurs as a component in amyloglucosidase preparations and can be obtained in substantially pure form from such preparations using an 35 appropriate separation technique, such as high performance liquid chromatography which is also the preferred method.

Although the novel acid amylase described below is obtained from a commercially available amyloglucosidase derived from the microorganism Aspergillus niger, and this is

the preferred amyloglucosidase, it will be appreciated that many genera of microorganisms contain species known to produce an amyloglucosidase. Any and all such amyloglucosidases can be used as the source of the novel acid amylase of this invention. Preferably, a fungal amyloglucosidase is used as the source.

The thermostability of the acid amylase derived from Aspergillus niger is better than that of the A. niger amyloglucosidase. Also the stability and residual activity of said acid amylase exceeds the same of said amyloglucosidase.

The invention further provides a novel enzyme product having both  $\alpha$ -1,4 and  $\alpha$ -1,6 bond splitting activity at acidic pH, which comprises amyloglucosidase and the novel acid amylase in a ratio of at least 0.16 AAU per AGI, as herein-after defined.

Such preparations may be made by adding the novel acid amylase to a known amyloglucosidase preparation so as to increase the acid amylase content of the latter.

Preferably, the amyloglucosidase is an Aspergillus niger amyloglucosidase and is enriched with the novel acid amylase also derived from Aspergillus niger.

The novel enzyme product of the invention can be prepared by adding the new acid amylase, preferably in substantially pure form, to the amyloglucosidase. Alternatively, an amyloglucosidase producing strain or variant or mutant thereof, preferably belonging to the genus Aspergillus and more preferably to the species A. niger, may be found which produces an amyloglucosidase with a relatively high acid amylase content as compared with the amyloglucosidases known in the art, in which case the enzyme product may be obtained by cultivating the said microorganism in a suitable nutrient medium containing carbon and nitrogen sources and inorganic salts. The novel enzyme product may also be prepared by selectively improving the fermentation conditions for acid amylase or partly inactivating the amyloglucosidase in existing preparations.

The amyloglucosidase used in this invention and also the novel enzyme product are preferably free from trans-glucosidase, since the latter enzyme may cause the formation

of undesired by-products. This can be effected e.g. by producing amyloglucosidase with a transglucosidase-negative strain or by removal of the transglucosidase from the amyloglucosidase preparations used, for example with bentonite.

5           The novel enzyme product of the invention comprises at least 0.16 AAU of acid amylase per AGI. One unit of acid amylase activity (AAU) as used herein is the amount of enzyme which hydrolyses 1.0 mg of soluble starch (100% of dry matter) per minute under standard conditions (pH 4.2; 60°C) into a  
10 product which, after reaction of an iodine solution of known strength, gives an optical density at 620 nm equivalent to that of a colour reference as described in the Iodine Starch Amylase Test described below. One unit of amyloglucosidase activity (AGI) as used herein is defined as the amount of  
15 enzyme that releases 1  $\mu$ mol of dextrose from soluble starch (100% of dry matter) per minute at 60°C under optimum conditions of starch degradation, as described hereinafter. Preferably, the novel enzyme product contains from about 0.2 to about 4.5 AAU of acid amylase per AGI, more preferably from  
20 about 0.3 to about 3.0 AAU per AGI and particularly from about 0.7 to about 1.5 AAU per AGI.

It has been surprisingly found that the amyloglucosidase preparations enriched with acid amylase, when used in the saccharification of liquefied starch, result in  
25 unexpectedly and significantly higher dextrose levels at shorter saccharification times. The results are comparable with those obtained by the simultaneous action of amyloglucosidase and acid pullulanase, as described in the aforementioned European Patent Application Publ. No. 0 063 909,  
30 under similar conditions.

Accordingly, the invention further provides a process for converting starch into dextrose in the form of a syrup, which comprises saccharifying the starch optionally and preferably after a liquefaction step to form a starch  
35 hydrolysate, in the presence of the novel enzyme product, as hereinbefore defined. The use of the new enzyme product in the process has the advantage that substantially lower amounts of amyloglucosidase can be used for saccharification of starch hydrolysates resulting in higher yields of glucose per enzyme

unit (AGI). The new enzyme product has also the great advantage that higher substrate concentrations can be used in the saccharification of starch and starch hydrolysates. The use of higher substrate concentrations substantially reduces 5 evaporation costs.

The saccharification is suitably carried out at a pH in the range of from 2.5 to 6, preferably of from about 3 to about 5 and more preferably of from about 4.0 to about 4.5. The process is suitably effected at temperatures in the range 10 of from 40 to 70°C, preferably of from about 50 to about 65°C, with reaction times in the range of from 15 to 96 hours to obtain maximum yields.

Preferred proportions of amyloglucosidase for the saccharification of starch hydrolysates are normally in the 15 range of from about 8 to about 30 AGI and preferably from about 14 to about 22 AGI per g of dry solids.

It has also been found that the saccharification of starch or a starch hydrolysate can be further improved, when the process is conducted in the presence of the novel enzyme 20 product as defined hereinbefore, which also contains an effective amount of acid pullulanase. A suitable acid pullulanase which can be used for the purpose of this invention is, for example, an acid pullulanase as described in European Patent Application Publ. No. 0 063 909. Preferred 25 dosages of acid pullulanase which can be used in conjunction with the novel enzyme product are in the range of from 0.005 to 5 pullulanase units (PU), the units being as defined in said European Patent Application. The use of the novel enzyme in conjunction with acid pullulanase in the process has the 30 advantage that unexpectedly and significantly high dextrose levels can be obtained at short saccharification times.

Another suitable method to determine the amount of acid amylase in enzyme preparations is the modified Phadebas Amylase Test described below. One unit of acid amylase 35 activity (AAU') as used herein is defined as the amount of enzyme that gives one unit of absorbance at 620 nm under modified Phadebas amylase test conditions described below. The value of at least 0.16 AAU of acid amylase per AGI under Iodine Starch Amylase Test conditions, as defined herein-

before, corresponds with the value of at least 0.12 AAU' of acid amylase per AGI under modified Phadebas Amylase Test conditions. A drawback of the latter method is a synergistic effect which occurs when amyloglucosidase is present.

5 Moreover, it is very difficult or even impossible to automatize this method.

It will be understood that, unless otherwise stated, the AAU values which are mentioned in this specification are expressed in units according to the modified Iodine Starch

10 Amylase Test method.

The following test methods and Examples illustrate the invention.

#### IODINE STARCH AMYLASE TEST

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This method is based on the measurement of the optical density of iodine starch complexes in the presence of an amyloglucosidase inhibitor. Acarbose, Bay g 5421, was used as the amyloglucosidase inhibitor, cf. Schmidt et al,

20 Naturwissenschaften 64 (1977) 535.

#### Reagents

- A 2% solution of soluble starch (Lintner, J.T. Baker Co.) in  
25 citrate buffer (0.013 M, pH 4.2).

- Iodine stock solution containing 22 g iodine and 44 g potassium iodide per litre of distilled water.

30 - Diluted iodine solution: 4 ml of iodine stock solution and 40 g potassium iodide dissolved in distilled water. Distilled water added up to 1 litre.

- Colour reference containing 250 g cobaltous chloride 6 aq.  
35 and 38.4 g potassium bichromate per litre in 0.01 N HCl.

#### Procedure

The starch solution (20 ml) was preheated at 60°C

for 20 min. Starting at time 0 exactly 10 ml of the enzyme sample (containing 1.4 - 1.8 AAU/ml; room temperature) was added to the substrate solution. If amyloglucosidase is believed to be present in the enzyme sample, the amylo-  
5 glucosidase inhibitor Bay g 5421 is previously added to the enzyme sample in a concentration of 1 ug per AGI. After 20 min. of incubation 1 ml of the solution was transferred to 5 ml of the diluted iodine solution. The optical density was immediately measured at 620 nm in a 1 cm cuvet using distilled  
10 water as the blank. This procedure of transferring and measuring was repeated at 1 min. intervals until readings were found which were lower than the readings of the colour reference.

15 The time T needed to reach the absorbance equal to that of the colour reference was established graphically.

The acid amylase activity in units (AAU) present in the incubation solution was calculated from  $400/T$  in which:  
20 400: mg of soluble starch in the incubation solution  
T : reaction time needed (min.)

#### MODIFIED PHADEBAS AMYLASE TEST

25 The standard Phadebas amylase test (Marciniak et al., Starch 34 442 (1982)) modified for conditions of acidic pH and a temperature of 60°C was effected as follows. In a glass vial with screwed cap 1 ml of enzyme sample containing 10 AGI and 4.0 ml acetate buffer (0.3M, pH 4.0) were pipetted.  
30 Then a Phadebas tablet (Pharmacia, batch no. HE 74112) was added and after vortexing for 15 sec. the tube was closed and placed in a water bath at 60°C. The reaction was stopped exactly 15 min. after the addition of the tablet by adding 0.3 N NaOH (5 ml) and shaking. After centrifugation the super-  
35 natant was removed and the optical density (OD) was measured (in the range 0.2 to 2.0) in a 1 cm cuvet at 620 nm relative to distilled water. A blank (distilled water) underwent the same procedure. The  $\Delta OD$  is a measure of the acid amylase activity. One unit of acid amylase activity (AAU') is defined

as the amount of enzyme that gives one unit of absorbance ( $\Delta OD = 1$ ) at 620 nm under these test conditions.

#### AMYLOGLucosidase Assay

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Soluble starch (2 ml; Lintner Starch, J.T. Baker Co.) in a concentration of 16 g/l of acetate buffer (0.04M, pH 4.3) was preheated at 60°C for 5 min. and then added to 2 ml of enzyme solution (0.15-0.55 AGI/ml). After mixing the  
10 suspension was incubated at 60°C. The reaction was terminated after 15 min. by adding 20 ml NaOH (0.005 N) and the glucose concentration determined by the glucose oxidase method.

#### THE SACCHARIFICATION PROCESS

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The saccharification process was effected on maltodextrin MD03 (Roquette Frères) having a dextrose equivalent (DE) of 16.5. This substrate contains some oligosaccharides having fructosyl end groups, from which as much as 0.4-0.5% of  
20 the disaccharide maltulose is formed in the saccharification step. To a solution of this substrate (33% dry solids) 2100 AGI/100 g dry solids were added. The pH was adjusted to 4.20 with 1 N acetic acid. The mixture was incubated at 60°C in a water bath. Aliquots of 0.1 ml were taken from the reaction  
25 mixture at 16, 24, 48, 64, 72, 80 and 92 hr and added to 3 ml of distilled water in a closed test tube.

Each diluted sample was immediately placed into a boiling water bath for 10 min. in order to inactivate the enzyme. After cooling about 150 mg of dried Amberlite MB-3  
30 resin (BDH) were added to each sample in order to remove HPLC disturbing salts. After standing for 1 hr the resin was removed and 40  $\mu$ l of sample were injected onto the HPLC for glucose determination according to the method of Scobell et al. (Cereal Chem., 54 (4), (1977) 905-917), modified in that a  
35 Bio-Rad HPX-87C 300 mm column was used. The precision and accuracy of the assay were found to be 0.1% and 0.2% absolute respectively at a glucose concentration in the range of 90-96%.

Under these conditions a peak level of 94.6-94.8% of

glucose was achieved using current commercial amyloglucosidase preparations from Miles (DIAZYME and OPTIDEX), Novo (AMG) and Gist-Brocades (AMIGASE GM).

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EXAMPLE IIsolation and Identification of Acid Amylase

In order to identify and isolate amylolytic components present in an amyloglucosidase preparation, an amyloglucosidase enzyme preparation produced by a trans-glucosidase negative strain of A. niger was subjected to high performance liquid chromatography. The system comprised an anion exchange column and a gel filtration column coupled in series. After injection of a part of the AG preparation onto the ion exchange column, the solvent, which was 0.05 M sodium acetate buffer with a pH of 4.0, was led through both columns until the positively charged and uncharged components had reached the gel column. The molecules adsorbed on the ion column were eluted by a salt gradient (0.05 - 1.65 M sodium acetate buffer, pH 4.0) and then the molecules bound on the gel column were eluted with the original solvent.

This procedure revealed an excellent separation as can be seen from the accompanying Fig. 1, between the amylolytic enzymes, and both amyloglucosidase isomers and an  $\alpha$ -amylase were identified by fractionating the effluent and incubating the collected fraction with suitable substrates, i.e. maltose and soluble starch (10% dry solids). The isolated amyloglucosidase components produced glucose from starch and maltose, whereas the isolated  $\alpha$ -amylase produced a typical oligosaccharide pattern from starch.

The characteristics of the  $\alpha$ -amylase, in particular in relation to pH and temperature, were determined using soluble starch as a substrate. The amounts of the main products formed (di- and trisaccharides) showed an optimum at a pH of 3.5 to 5.0 indicating that the enzyme is a true acid amylase (AA). The effect of temperature was investigated at a pH of 4.0 and the acid amylase had its optimum between 65 and 70°C as was determined from the behaviour of the tri-

saccharides formed. These results indicate that the acid amylase is sufficiently stable at the standard saccharification temperature of 60°C. The fractions containing AA, the activity of which was stable for more than 3 months, 5 were used for enrichment experiments.

### EXAMPLE II

#### Saccharification with Acid Amylase Enriched Samples

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Saccharification was accomplished on maltodextrin MDO3 having a dextrose equivalent (DE) of 16.5, and, to a solution of this substrate (33% dry solids), 2100 AGI/100 g dry solids were added. Also, various amounts of acid amylase 15 were added, the activity of which was previously determined according to Phadebas method described above. During the saccharification, the starch hydrolysate was maintained at a pH of 4.0-4.2 and a temperature of 60°C. Under these conditions the degree of saccharification or glucose formation 20 was measured over the period between 17 and 91 hrs.

The experiments with acid amylase enriched samples which were completely free of transglucosidase demonstrated that the glucose yield was increased and the saccharification time was shortened as can be seen from the results of Table I below.

TABLE I

Sample No.	AAU'/AGI			Glucose yield (%)						
	(a)	(b)	(c)	Saccharification time in hr.						
				17	24	41	48	65	72	91
1	0.08	--	0.074	89.1	92.5	94.1	94.1	94.7	94.7	94.5
2	0.08	0.07	0.20	90.7	93.6	94.6	94.8	94.7	94.7	94.5
3	0.08	0.15	0.35	91.7	94.2	94.9	94.8	95.0	94.8	94.6
4	0.08	0.35	0.73	92.7	94.6	94.9	94.9	94.6	94.8	94.5
5	0.08	0.70	1.40	93.5	95.0	94.8	94.8	95.0	94.8	94.2
6	0.08	1.50	2.92	94.0	94.9	94.8	--	94.4	94.4	94.3
7	0.08	2.32	4.48	94.4	95.1	94.7	--	95.0	94.5	94.4

(a): present in original amyloglucosidase preparation (measured with modified Phadebas method)

(b): AAU' of added acid amylase (measured with modified Phadebas method)

(c): acid amylase activities converted into AAU (as defined hereinbefore in the Iodine Starch Amylase Test)

The results of Table I show that acid amylase increases the yield of glucose from 94.7% to 95.1% under these conditions while the saccharification time for optimum yields decreased from 70 hours to 24 hours. This makes the use of acid amylase commercially important and equivalent to the results obtained with pullulanase. A portion of the amyloglucosidase can be replaced with the acid amylase while obtaining economically attractive glucose yields and saccharification times.

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### EXAMPLE III

Using the saccharification procedure of Example I, tests were run with the normal and one half normal dosages of amyloglucosidase and with one half normal dosages of amyloglucosidase with enrichment of acid amylase and acid pullulanase, described in European Patent Application Publication No. 0 063 909, alone and in combination. 1 pullulanase unit (PU) is defined as the amount of enzyme necessary to produce 1  $\mu$ mole of reducing sugar from pullulan per minute under standard conditions. The results are reported in Table II.

TABLE II

Sample No.	Glucose yield (%)				
	AGI (relative dosage)	AAU' / AGI (a)	AAU' / AGI (b)	AAU / AGI (c)	PU/gDS
					Saccharification time in hr.
					17 24 43.5 48 65 71 89
1	1.0	0.08	--	0.074	90.8 92.3 94.3 94.4 94.6 94.7 94.7
2	0.5	0.08	--	0.074	80.1 87.0 91.0 91.8 93.1 93.7 94.4
3	0.5	0.08	0.88	1.74	85.4 91.4 94.6 94.5 95.0 95.0 95.3
4	0.5	0.08	0.88	1.74	87.3 93.5 95.5 95.4 95.5 95.7 95.5
5	0.5	0.08	1.68	3.26	88.1 93.3 94.9 95.0 95.1 95.4 95.6
6	0.5	0.08	--	--	83.4 90.4 95.2 95.4 95.5 95.5 95.7
7	0.5	0.08	1.68	3.26	90.3 94.2 95.4 95.4 95.4 95.3 95.5

(a): present in original amyloglucosidase preparation (measured with modified Phadebas method)

(b): AAU' of added acid amylase (measured with modified Phadebas method)

(c): acid amylase activities converted into AAU (as defined hereinbefore in the Iodine Starch Amylase Test)

The results of Table II show that the glucose level reached its peak after about 80 hours using half the normal amyloglucosidase (AG) and an acid amylase (AA) enrichment factor, which is slightly longer than the 70 hour saccharification time using the normal amyloglucosidase dosage. However, the peak level of glucose increased from 94.7% to 95.5% which may be due to less isomaltose formation consequent upon the smaller amount of amyloglucosidase used.

Similar results were obtained when pullulanase and amyloglucosidase were used together, although significant differences in glucose production were noted at the shorter saccharification times. The combination of acid amylase, pullulanase and one half of amyloglucosidase showed a faster saccharification resulting in higher yields of glucose per enzyme activity (AGI) per hour.

These results indicate that acid amylase substantially contributes to the hydrolysis of starch in the saccharification step. This surprising effect competes with that of the acidic pullulanase, although the two enzymes act by basically different mechanisms. While pullulanase is thought to be an endo  $\alpha$ -1,6 bond splitter, acid amylase has  $\alpha$ -1,4 bond splitting activity.

#### EXAMPLE IV

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Using the novel acid amylase in the saccharification of starch makes possible an increase in the glucose peak levels, a shortening of the saccharification times and a reduction of the necessary amyloglucosidase/dry solids (DS) ratio. Another advantage in using acid amylase in the saccharification of liquefied starch is the increase in substrate concentration which is then possible, which can substantially reduce evaporation costs.

Solutions containing substrate (MDO3) in various dry matter contents were adjusted to pH 4.2 and heated to 60°C. Half normal AG dosages (10.5 AGI/gDS) and a 9-fold amount of acid amylase were added. Aliquots were taken at various intervals and analyzed as described in Example I. Control experiments with normal and halved AG dosages without

additional acid amylase were also carried out. The results are given in Table III below.

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TABLE III

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%DS*)	AGI/gDS	AAU/AGI	Saccharification time (h)	maximum glucose(%)
25	10.5	0.074	140-165	95.6
25	10.5	0.74	64	96.1
29	10.5	0.074	140-165	95.3
29	10.5	0.74	90	95.8
33	10.5	0.074	140-165	94.6
33	10.5	0.74	90	95.2
37	10.5	0.074	140-165	93.9
37	10.5	0.74	71	94.7
45	10.5	0.074	140-165	91.3
45	10.5	0.74	71	92.8
33	21	0.074	71	94.7

20 \*) maltodextrin MD03

The data in Table III show that when amyloglucosidase is used in conjunction with the new acid amylase the dry matter content (DS) can be elevated to yield maximum glucose levels which are higher than those obtained under similar conditions using commercial amyloglucosidase preparations. For example, a glucose peak level of 94.7% was obtained with a commercial amyloglucosidase preparation at 33% DS. The same maximum glucose level was achieved at the same incubation time with a 10 fold addition of acid amylase and half the amount of AG at 37% DS.

EXAMPLE V

35

Acid  $\alpha$ -amylases from other sources, i.e. bacterial enzymes, which are active in the acidic pH range and at 60°C, can also be used to improve the saccharification brought about by amyloglucosidase. Thus a crude fermentation sample of the bacterium ATCC 31199 (see British Specification No. 1539694

CPC International), containing  $\alpha$ -amylase activity, was used in a saccharification experiment with amyloglucosidase. Using the crude sample in a ratio of AAU/AGI = 0.74 gave significant higher glucose levels compared with those obtained with amyloglucosidase only in a control experiment, although the values were lower than those obtained by a corresponding amount of the fungal acid amylase. The results are shown in the following Table IV.

TABLE IV

			Glucose yield (%)									
Sample	AAU/AGI	AGI/gDS	Saccharification time in hr.									
			24	40	47	64	71	88	95	112		
AG	0.074	10.5	80.2	88.9	90.3	92.2	92.8	93.6	94.0	94.3		
AG + Bacterial AA	0.74	10.5	81.7	90.4	91.6	93.1	93.5	94.1	94.2	94.6		
AG + Fungal AA (cf. Example 1)	0.74	10.5	--	94.4	94.7	95.1	95.2	95.2	--	--		

EXAMPLE VI

Saccharification experiments were performed by the same procedure as described hereinbefore. Solutions containing substrate (MDO3, 33% DS) were adjusted to pH values between 3.5 and 5.0 and heated to 60°C. Amyloglucosidase (21 AGI/g DS) together with a 9-fold amount of AA (compared with the amount present in the AG preparation) were added. Aliquots were taken at various intervals and analyzed. Control experiments were also performed. The following data were obtained, see Table V.

TABLE V

	Starting pH	pH after 94 h	saccharification time (h)	glucose peak level (%)
15	3.5	3.45	71	95.3
	4.0	3.85	64	95.3 (94.9)*
	4.2	3.95	64	95.3 (94.8)*
	4.5	4.1	64	95.3 (94.6)*
20	5.0	4.2	64	95.2

\* Controls (AG dosages without extra AA addition)

Thus, using excess of acid amylase comparable glucose peak levels were obtained in the pH range of 3.5 to 5.0.

EXAMPLE VII

Solutions containing substrate (MDO3, 33% DS) were adjusted to pH 4.2 and heated to various temperatures. Amyloglucosidase (21 AGI/gDS) and a 9-fold amount of acid amylase were added. Aliquots were taken and analyzed as described in Example II. Controls (AG dosages without extra AA addition) were also carried out. The results are given in Table VI below.

TABLE VI

	Temperature	AAU/AGI	saccharification time in hr.	glucose yield (%)
5	55	0.074	65	94.6
		0.74	47	95.0
	57.5	0.074	71	95.0
		0.74	47	95.5
	60	0.074	71	94.8
10		0.74	64	95.3
	62.5	0.074	90	94.8
		0.74	64	95.4
	65	0.074	117	93.0
		0.74	89	94.6

15

These results confirm that acid amylase is stable at temperatures up to at least 65°C, which makes it very suitable for use in conjunction with amyloglucosidase at relatively high saccharification temperatures. The lower glucose values at 65°C are likely caused by the lower thermostability of the AG enzyme relative to AA. The presence of acid amylase has a beneficial effect on the glucose production at higher temperature.

25

CLAIMS

1. A microbial acid amylase having substantially  $\alpha$ -1,4-glucosidic bond splitting activity.
- 5           2. A microbial acid anylase showing optimum saccharification at a pH from 3.5 to 5.0 at a temperature from 60 to 75°C.
3. The acid amylase of claim 1 or 2 which is derived from a fungus.
- 10           4. The acid amylase of claim 3 which is derived from Aspergillus niger.
5. An enzyme product comprising amyloglucosidase and acid amylase as defined in any one of claims 1 to 4 in a ratio of at least 0.16 AAU per AGI, as hereinbefore defined.
- 15           6. An enzyme product according to claim 5 containing 0.2 to 4.5 AAU per AGI.
7. An enzyme product according to claim 5 or 6 wherein the amyloglucosidase is derived from Aspergillus niger.
- 20           8. An enzyme product according to any one of claims 5 to 7, substantially free of transglucosidase.
9. An enzyme product according to any one of claims 5 to 8 also containing an effective amount of acid pullulanase.
- 25           10. An enzyme product according to claim 9 wherein the acid pullulanase is a pullulanase as described in European Patent Application Publ. No. 0 063 909.
11. A process for converting starch into dextrose in the form of a syrup which comprises saccharifying starch or a  
30 starch hydrolysate in the presence of an enzyme product as defined in any one of claims 5 to 10.
12. A process according to claim 11 wherein a starch hydrolysate containing at least 30% by weight of dry solids is saccharified.
- 35           13. A process according to claim 11 or 12 wherein the saccharification is conducted in the pH-range of from 3 to 5 and at a temperature in the range of from 40 to 70°C.
14. A process according to claim 13 wherein the

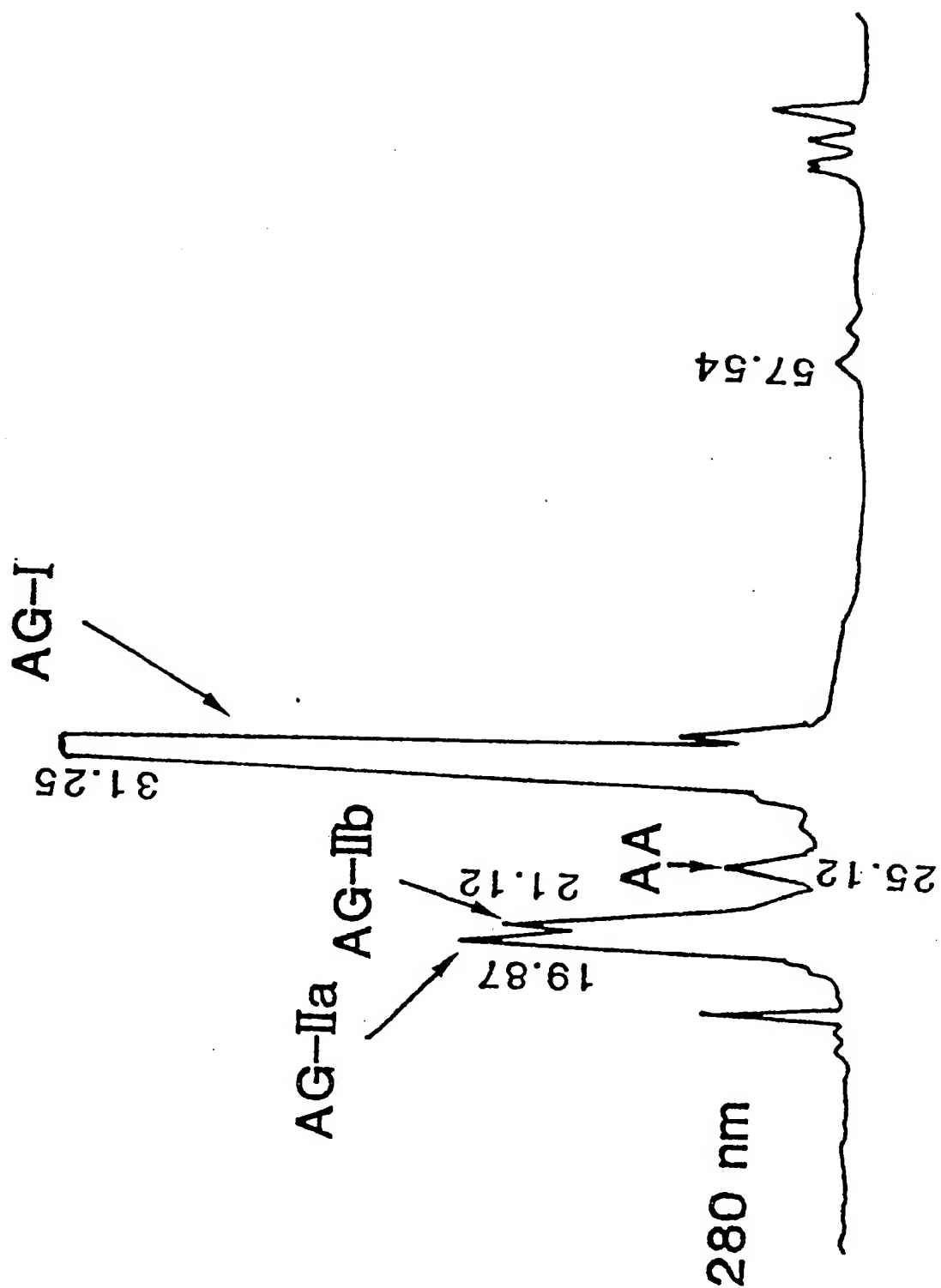
saccharification is conducted at pH 4 to 4.5 at a temperature of 50 to 65°C.

15. A process according to any of claims 11 to 14 wherein the amount of amyloglucosidase used is from 8 to 30 5 AGI per g of total dry solids.

16. A process according to claim 15 wherein the amount of amyloglucosidase used is from 14 to 22 AGI per g of total dry solids.

17. A process according to any one of claims 11 to 16 10 wherein the saccharification is conducted in the presence of acid pullulanase.

FIG. I





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Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
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The present search report has been drawn up for all claims			TECHNICAL FIELDS SEARCHED (Int. Cl. 4)
Place of search THE HAGUE		Date of completion of the search 13-12-1984	Examiner DEKEIREL M.J.
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons &amp; : member of the same patent family, corresponding document</p>			



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Page 2

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The present search report has been drawn up for all claims				
Place of search THE HAGUE		Date of completion of the search 13-12-1984	Examiner DEKEIREL M.J.	
<p><b>CATEGORY OF CITED DOCUMENTS</b></p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons &amp; : member of the same patent family, corresponding document</p>				



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DOCUMENTS CONSIDERED TO BE RELEVANT				Page 3
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)	
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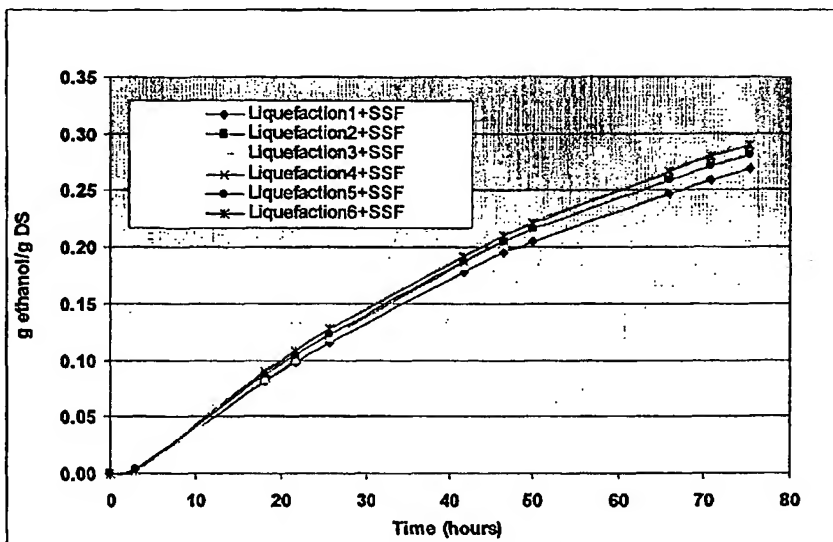
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(54) Title: LIQUEFACTION PROCESS



(57) Abstract: The present invention relates to method of liquefying starch-containing material, wherein the method comprises the steps of (a) treating the starch-containing material with a bacterial alpha-amylase at a temperature around 70-90° C for 15-90 minutes, and (b) treating the material obtained in step (a) with an alpha-amylase at a temperature between 60-80° C for 30-90 minutes. The invention also relates to a process of producing a fermentation product, preferably ethanol, comprising a liquefaction step carried out according to the liquefaction method of the invention.



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## LIQUEFACTION PROCESS

### CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority from U.S. provisional application serial nos. 5 60/575,133, filed on May 28, 2004, and 60/554,615, filed on March 19, 2004, which are hereby incorporated by reference. This application contains a sequence listing, which is hereby incorporated by reference.

### FIELD OF THE INVENTION

10 The present invention relates to an improved method of liquefying starch-containing material suitable as step in processes for producing syrups and fermentation products, such as especially ethanol. The invention also relates to processes for producing a desired fermentation product, preferably ethanol, comprising liquefying starch-containing starting material in accordance with the liquefaction method of the invention.

15

### BACKGROUND OF THE INVENTION

Liquefaction is a well known process step in the art of producing syrups and fermentation products, such as ethanol, from starch-containing materials. During liquefaction starch is converted to shorter chains and less viscous dextrans. Generally liquefaction involves gelatinization of starch simultaneously with or followed by addition of alpha-amylase. 20

WO 02/38787 (Novozymes) disclose a method of producing ethanol by fermentation comprising carrying out secondary liquefaction in the presence of a thermostable acid alpha-amylase or a thermostable maltogenic acid alpha-amylase.

Even though liquefaction has already been improved significantly there is still a 25 need for improving liquefaction suitable for syrup and fermentation product producing processes.

### SUMMARY OF THE INVENTION

The object of the present invention is to provide an improved method of liquefying 30 starch-containing material suitable as a step in processes for producing syrups and fermentation products, such as especially ethanol. The invention also provides a process for producing a desired fermentation product which includes a liquefaction method of the invention.

The present inventors have found that when liquefaction is carried out on starch-containing material in accordance with the present invention a number of advantages are 35 obtained. For instance, the inventors have shown that a DE above 20 may be obtained without using more enzyme than corresponding prior art processes which reaches a DE around 12. Further, reduced viscosity was observed. This eases handling of the liquefied material and reduces the cost of pumping the liquefied material to down stream process equipment

such as a fermentor. Furthermore, the enzyme cost is also reduced. It was also found that the sugar profile of the liquefied mash had a decreased DP<sub>4+</sub> content and increased DP<sub>1-3</sub> content compared to corresponding prior art methods using higher amounts of enzyme. The higher DP<sub>1-3</sub> content makes the liquefied mash easier and potentially faster to ferment by a fermenting organism, such as yeast, during, e.g., ethanol fermentation. This could be attributed to the fact that small sugars are released pertaining to the (acid) alpha-amylase action. These small sugars, e.g., glucose, maltose and maltotriose, can be directly metabolized by the fermenting organism and therefore makes SSF more effective and fast.

Further, also the residual DP<sub>4+</sub> content were after fermentation found to be higher than in corresponding prior art processes. This indicates a better utilization of the starch-containing starting material.

The abbreviation "DE" stands for "Dextrose Equivalent" and is a measure for reducing ends on C<sub>6</sub> carbohydrates. Pure dextrose (glucose) has a DE of 100. Dextrose is a reducing sugar. Whenever an amylase hydrolyzes a glucose-glucose bond in starch, two new glucose end-groups are exposed. At least one of these can act as a reducing sugar. Therefore the degree of hydrolysis can be measured as an increase in reducing sugars. The value obtained is compared to a standard curve based on pure glucose - hence the term dextrose equivalent. In other words: DE (dextrose equivalent) is defined as the amount of reducing carbohydrate (measured as dextrose-equivalents) in a sample expressed as w/w% of the total amount of dissolved dry matter.

According to the first aspect the invention relates to a method of liquefying starch-containing material, wherein the method comprises the steps of:

- (a) treating starch-containing material with a bacterial alpha-amylase at a temperature around 70-90°C for 15-90 minutes,
- (b) treating the material obtained in step (a) with an alpha-amylase at a temperature between 60-80°C for 30-90 minutes.

The term "mash" is used for liquefied starch-containing material, such as liquefied whole grain.

In one embodiment of the invention the starch-containing material is jet-cooking at 90-120°C, preferably around 105°C, for 1-15 minutes, preferably for 3-10 minute, especially around 5 minutes, before step (a).

After step (b) the mash has a DE value above 16, preferably above 18, especially above 20, such as a DE value in the range from 16 to 30, preferably in the range from 18 to 25.

In a second aspect the invention provides a process of producing a fermentation product, especially ethanol, from starch-containing material by fermentation, said process comprises the steps of:

- 5 (i) liquefying starch-containing material according to the liquefaction method of the invention;
- (ii) saccharifying the liquefied mash obtained;
- (iii) fermenting.

Optionally the ethanol is recovery after fermentation. In an embodiment the saccharification and fermentation is carried out as a simultaneous saccharification and fermentation process (SSF process).

## BRIEF DESCRIPTION OF THE INVENTION

**Fig. 1:** Ethanol yields from six liquefaction treatments with 0.3 AGU/g DS of Glucoamylase SF.

## DESCRIPTION OF THE INVENTION

The present invention provides an improved liquefaction method suitable as a step in processes for producing fermentation products such as especially ethanol. The invention also relates to a process of producing a fermentation product, especially ethanol, comprising a liquefaction method of the invention. Where the end product is ethanol it may be used as, e.g., fuel ethanol; drinking ethanol, i.e., potable neutral spirits; or industrial ethanol.

### Liquefaction

According to the present invention "liquefaction" is a process step in which starch-containing material, preferably milled (whole) grain, is broken down (hydrolyzed) into malto-

dextrins (dextrins).

Initially an aqueous slurry containing preferably from 10-40 wt-%, especially 25-35 wt-% starch-containing material is prepared. The starch-containing material is preferably milled whole grain. Then the starch-containing material is incubated with a bacterial alpha-amylase, preferably one or more *Bacillus* alpha-amylases, and may in one embodiment be followed by a jet-cooking step carried out between 90-120°C, preferably around 105°C, for 1-15 minutes, preferably for 3-10 minutes, especially around 5 minutes, to complete gelatinization of the slurry. However, it is to be understood that the method of the invention may also be carried out without a jet-cooking step. After incubation with bacterial alpha-amylase, with or without jet-cooking, the temperature is adjusted to 60-80°C and the material is incubated

for 30 to 90 minutes in the presence of an alpha-amylase, preferably an acid alpha-amylase, especially a fungal acid alpha-amylase, to finalize hydrolysis (secondary liquefaction).

Consequently, in the first aspect the invention provides a method for liquefying starch-containing material comprising the steps of:

- 5 (a) treating starch-containing material with a bacterial alpha-amylase at a temperature around 70-90°C for 15-90 minutes,
- (b) treating the material obtained in step (a) with an alpha-amylase at a temperature between 60-80°C for 30-90 minutes.

10 A liquefaction method of the invention is typically carried out at pH 4.5-6.5, in particular at a pH between 5 and 6.

The alpha-amylase may be any alpha-amylase, preferred an alpha-amylase mentioned in the section "Alpha-amylases" below.

#### Starch-containing material

15 The starch-containing material used according to the present invention may be selected from the group consisting of: tubers, roots and whole grain, and any combinations of the foregoing. In an embodiment, the starch-containing material is obtained from cereals. The starch-containing material may, e.g., be selected from the groups consisting of corns, cobs, wheat, barley, cassava, sorghum, rye, milo and potatoes; or any combination of the foregoing.

20 If the liquefaction method of the invention is included in an ethanol process of the invention, the raw starch-containing material is preferably whole grain or at least mainly whole grain. A wide variety of starch-containing whole grain crops may be used as raw material including: corn (maize), milo, potato, cassava, sorghum, wheat, and barley. Thus, in one embodiment, the starch-containing material is whole grain selected from the group consisting of corn (maize), milo, potato, cassava, sorghum, wheat, and barley; or any combinations thereof. In a preferred embodiment, the starch-containing material is whole grain selected from the group consisting of corn, wheat and barley or any combinations thereof.

25 The raw material may also consist of or comprise a side-stream from starch processing, e.g., C<sub>6</sub> carbohydrate containing process streams that are not suited for production of syrups.

30

#### Milling

35 In a preferred embodiment of the invention the starch-containing material is milled before step (a), i.e., before the primary liquefaction. Thus, in a particular embodiment, the liquefaction method further comprises - prior to the primary liquefaction step (i.e., prior to step (a), - the steps of:

- i. milling of the starch-containing material, such as whole grain;
- ii. forming a slurry comprising the milled starch-containing material and water.

The starch-containing material, such as whole grain, is milled in order to open up the structure and allowing for further processing. Two processes of milling are normally used in ethanol production processes: wet and dry milling. The term "dry milling" denotes milling of the whole grain. In dry milling the whole kernel is milled and used in the remaining part of the process. Wet milling gives a good separation of germ and meal (starch granules and protein) and is with a few exceptions applied at locations where there is a parallel production of syrups. Dry milling is preferred in processes aiming at producing ethanol. The term "grinding" is also understood as milling. In a preferred embodiment of the invention dry milling is used. However, it is to be understood that other methods of reducing the particle size of the starch-containing material are also contemplated and covered by the scope of the invention.

#### **Process for producing a fermentation product**

A process of the invention generally involves the steps of liquefaction, saccharification, fermentation and optionally recovering the fermentation product, such as ethanol, preferably by distillation.

According to this aspect, the invention relates to a process of producing a fermentation product, preferably ethanol, from starch-containing material by fermentation, said method comprises the steps of:

- (i) liquefying said starch-containing material according to the liquefaction method of the invention;
- (ii) saccharifying the liquefied mash obtained in step (i)
- (iii) fermenting.

In an embodiment the saccharification and fermentation steps ii) and iii) are carried out as a simultaneous saccharification and fermentation process (SSF process). In a preferred embodiment of the invention starch-containing raw material, such as whole grain, preferably corn, is dry milled in order to open up the structure and allow for further processing. The mash has before step (ii), i.e., after step (i), with or without jet-cooking before step i), a DE value of above 16, preferably above 18, especially above 20, such as a DE value in the range from 16 to 30, preferably in the range from 18 to 25.

A specific embodiment of the process of the invention comprises the steps of;

- 1) liquefying starch-containing material in accordance with the liquefaction method of the invention;

2) liquefying the material obtained in step 1) in the presence of an alpha-amylase having an amino acid sequence which has at least 70% identity to SEQ ID NO:1; and

3) saccharifying the material obtained; and

5 4) fermenting to produce a fermentation product, preferably ethanol;

wherein the steps 1), 2), 3) and 4) is performed in the order 1), 2), 3), 4) or wherein 4) is performed simultaneously with or following 3).

In a preferred embodiment a jet-cooking step, as defined above, is included before step 1). In a preferred embodiment the alpha-amylase used in step ii) is at least 75%, 80%,  
10 85% or at least 90%, e.g., at least 95%, at least 97%, at least 98%, or at least 99% identity to SEQ ID NO:1.

The mash has after step 2), with or without jet-cooking before step 1), a DE value of above 16, preferably above 18, especially above 20, such as a DE value in the range from 16 to 30, preferably in the range from 18 to 25.

15

#### Saccharification

"Saccharification" is a step in which the maltodextrin (such as, product from the liquefaction) is converted to low molecular sugars DP<sub>1-3</sub> (i.e., carbohydrate source) that can be metabolized by a fermenting organism, such as, yeast. Saccharification is well known in  
20 the art and is typically performed enzymatically using at least a glucoamylase or one or more carbohydrate-source generating enzymes as will be defined below. The saccharification step comprised in the process for producing ethanol of the invention may be a well known saccharification step in the art. In one embodiment glucoamylase, alpha-glucosidase and/or acid  
25 alpha-amylase is used for treating the liquefied starch-containing material. A full saccharification step may last up to from 20 to 100 hours, preferably about 24 to about 72 hours, and is often carried out at temperatures from about 30 to 65°C, and at a pH between 4 and 6, normally around pH 4.5-5.0. However, it is often more preferred to do a pre-saccharification step, lasting for about 40 to 90 minutes, at temperature of between 30-65°C, typically about  
30 60°C, followed by complete saccharification during fermentation in a simultaneous saccharification and fermentation process (SSF). The most widely used process for ethanol production is the simultaneous saccharification and fermentation (SSF) process, in which there is no holding stage for the saccharification, meaning that fermenting organism, such as yeast, and enzyme(s) is(are) added together. In SSF processes, it is common to introduce a pre-saccharification step at a temperature between 40 and 60°C, preferably around 50°C, just  
35 prior to the fermentation.

#### Fermentation Product

The term "fermentation product" means a product produced by a process including a fermentation step using a fermenting organism. Fermentation products contemplated according to the invention include alcohols (e.g., ethanol, methanol, butanol); organic acids (e.g., citric acid, acetic acid, itaconic acid, lactic acid, gluconic acid); ketones (e.g., acetone); amino acids (e.g., glutamic acid); gases (e.g., H<sub>2</sub> and CO<sub>2</sub>); antibiotics (e.g., penicillin and tetracycline); en-zymes; vitamins (e.g., riboflavin, B12, beta-carotene); and hormones. In a preferred embodiment the fermentation product is ethanol, e.g., fuel ethanol; drinking ethanol, i.e., potable neutral spirits; or industrial ethanol or products used in the consumable alcohol industry (e.g., beer and wine), dairy industry (e.g., fermented dairy products), leather industry and tobacco industry. Preferred beer types comprise ales, stouts, porters, lagers, bitters, malt liquors, happoushu, high-alcohol beer, low-alcohol beer, low-calorie beer or light beer. Preferred fermentation processes used include alcohol fermentation processes, as are well known in the art. Preferred fermentation processes are anaerobic fermentation processes

15

#### Fermentation

In a process of the invention the fermenting organism is preferably yeast, which may be applied to the saccharified material.

The term "fermenting organism" refers to any organism suitable for use in a desired fermentation process. Suitable fermenting organisms are according to the invention capable to ferment, i.e., convert sugars, such as glucose or maltose, directly or indirectly into the desired fermentation product, preferably ethanol. Examples of fermenting organisms include fungal organisms, such as yeast. For ethanol production preferred yeast includes strains of *Saccharomyces* spp., and in particular *Saccharomyces cerevisiae*. Commercially available yeast includes, e.g., RED STAR®/Lesaffre Ethanol Red (available from Red Star/Lesaffre, USA) FALI (available from Fleischmann's Yeast, a division of Burns Philp Food Inc., USA), SUPERSTART (available from Alltech), GERT STRAND (available from Gert Strand AB, Sweden) and FERMIOL (available from DSM Specialties). In preferred embodiments, yeast is applied to the saccharified mash. Fermentation is ongoing for 24-96 hours, such as typically 35-65 hours. In preferred embodiments, the temperature is generally between 26-34°C, in particular about 32°C, and the pH is generally from pH 3-6, preferably around pH 4-5. Yeast cells are preferably applied in amounts of 10<sup>5</sup> to 10<sup>12</sup>, preferably from 10<sup>7</sup> to 10<sup>10</sup>, especially 5x10<sup>7</sup> viable yeast count per ml of fermentation broth. During the ethanol producing phase the yeast cell count should preferably be in the range from 10<sup>7</sup> to 10<sup>10</sup>, especially around 2 x 10<sup>8</sup>. Further guidance in respect of using yeast for fermentation can be found in, e.g., "The alcohol Textbook" (Editors K. Jacques, T.P. Lyons and D.R.Kelsall, Nottingham University Press, United Kingdom 1999), which is hereby incorporated by reference.

35

### Recovery of ethanol

Optionally the ethanol is recovery after fermentation, preferably by including the step of;

(iv) distillation to obtain the ethanol;

wherein the fermentation in step (iii) and the distillation in step (iv) is carried out simultaneously or separately/sequential; optionally followed by one or more process steps for further refinement of the ethanol.

### Starch Conversion

The liquefaction method of the invention may also be included in a starch conversion process for producing syrup such as glucose, maltose, fructose syrups, e.g., high fructose syrup (HFS), malto-oligosaccharides and isomalto-oligosaccharides. Suitable starting materials are exemplified in the "Starch-containing material"-section above. The process comprises a liquefaction method of the invention followed by saccharification in order to, e.g., release sugar from the non-reducing ends of the starch or related oligo- and polysaccharide molecules in the presence of carbohydrate-source generating enzyme.

Consequently, this aspect of the invention relates to a process of producing syrup from starch-containing material, comprising

- (a) liquefying starch-containing material in accordance with the liquefaction method of the invention,
- (b) saccharifying the liquefied material.

To produce, e.g., fructose an isomerization step is included. Optionally the syrup may be recovered from the saccharified material obtained in step (b) or after an additional step.

Details on suitable liquefaction and saccharification conditions can be found above.

### **Alpha-amylases**

According to the invention preferred any alpha-amylases may be used. Preferred alpha-amylases are of fungal or bacterial origin.

### Bacterial alpha-amylase

The bacterial alpha-amylase may be any bacterial alpha-amylase.

In a preferred embodiment the *Bacillus* alpha-amylase is derived from a strain of *B. licheniformis*, *B. amyloliquefaciens*, *B. subtilis* or *B. stearothermophilus*, but may also be derived from other *Bacillus* sp. Specific examples of contemplated alpha-amylases include the *Bacillus licheniformis* alpha-amylase (BLA) shown in SEQ ID NO: 4 in WO 99/19467, the *Bacillus amyloliquefaciens* alpha-amylase (BAN) shown in SEQ ID NO: 5 in WO 99/19467, and the *Bacillus stearothermophilus* alpha-amylase (BSG) shown in SEQ ID NO: 3 in WO 99/19467. In an embodiment of the invention the alpha-amylase is an enzyme having a de-

gree of identity of at least 60%, preferably at least 70%, more preferred at least 80%, even more preferred at least 90%, such as at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identity to any of the sequences shown as SEQ ID NOS: 1, 2, 3, 4, or 5, respectively, in WO 99/19467. Other alpha-amylases include alpha-amylase derived from a strain of the *Bacillus* sp. NCIB 12289, NCIB 12512, NCIB 12513 or DSM 9375, all of which are described in detail in WO 95/26397, and the alpha-amylase described by Tsukamoto et al., Biochemical and Biophysical Research Communications, 151 (1988), pp. 25-31.

The *Bacillus* alpha-amylase may also be a variant and/or hybrid, especially one described in any of WO 96/23873, WO 96/23874, WO 97/41213, WO 99/19467, WO 00/60059, and WO 02/10355 (all documents hereby incorporated by reference). Specifically contemplated alpha-amylase variants are disclosed in US patent nos. 6,093,562, 6,297,038 or US patent no. 6,187,576 (hereby incorporated by reference) and include *Bacillus stearothermophilus* alpha-amylase (BSG alpha-amylase) variants having a deletion of one or two amino acid in positions R179 to G182, preferably a double deletion disclosed in WO 1996/023873 – see e.g., page 20, lines 1-10 (hereby incorporated by reference), preferably corresponding to delta(181-182) compared to the wild-type BSG alpha-amylase amino acid sequence set forth in SEQ ID NO:3 disclosed in WO 99/19467 or deletion of amino acids R179 and G180 using SEQ ID NO:3 in WO 99/19467 for numbering (which reference is hereby incorporated by reference). Even more preferred are *Bacillus* alpha-amylases, especially *Bacillus stearothermophilus* alpha-amylase, which have a double deletion corresponding to delta(181-182) and further comprise a N193F substitution (also denoted I181\* + G182\* + N193F) compared to the wild-type BSG alpha-amylase amino acid sequence set forth in SEQ ID NO:3 disclosed in WO 99/19467.

A hybrid alpha-amylase specifically contemplated comprises 445 C-terminal amino acid residues of the *Bacillus licheniformis* alpha-amylase (shown as SEQ ID NO: 4 in WO 99/19467) and the 37 N-terminal amino acid residues of the alpha-amylase derived from *Bacillus amyloliquefaciens* (shown as SEQ ID NO: 3 in WO 99/19467), with one or more, especially all, of the following substitution:

G48A+T49I+G107A+H156Y+A181T+N190F+I201F+A209V+Q264S (using the *Bacillus licheniformis* numbering). Also preferred are variants having one or more of the following mutations (or corresponding mutations in other *Bacillus* alpha-amylase backbones): H154Y, A181T, N190F, A209V and Q264S and/or deletion of two residues between positions 176 and 179, preferably deletion of E178 and G179 (using the SEQ ID NO: 5 numbering of WO 99/19467). The bacterial alpha-amylase may be added in an amount well-known in the art. When measured in KNU units the alpha-amylase activity is preferably present in an amount

of 0.5-5,000 NU/g of DS, in an amount of 1-500 AAU/kg of DS, or more preferably in an amount of 5-1,000 KNU/kg of DS, such as 10-100 KNU/kg DS.

#### Fungal alpha-amylase

5 The fungal alpha-amylase may be any fungal alpha-amylase. Preferred fungal alpha-amylases include alpha-amylases derived from a strain of *Aspergillus*, such as, *Aspergillus oryzae*, *Aspergillus niger*, or *A. kawashii* alpha-amylases. In a preferred embodiment, the alpha-amylase is an acid alpha-amylase. In a more preferred embodiment the acid alpha-amylase is an acid fungal alpha-amylase or an acid bacterial alpha-amylase. More preferably, the acid alpha-amylase is an acid fungal alpha-amylase derived from the genus *Aspergillus*. A commercially available acid fungal amylase is SP288 (available from Novozymes A/S, Denmark).

15 In an embodiment the alpha-amylase is an acid alpha-amylase. The term "acid alpha-amylase" means an alpha-amylase (E.C. 3.2.1.1) which added in an effective amount has activity at a pH in the range of 3.0 to 7.0, preferably from 3.5 to 6.0, or more preferably from 4.0-5.0.

A preferred acid fungal alpha-amylase is a Fungamyl-like alpha-amylase. In the present disclosure, the term "Fungamyl-like alpha-amylase" indicates an alpha-amylase which exhibits a high identity, i.e., more than 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% 90%, 95 or even 99% identical to the amino acid sequence shown in SEQ ID NO: 10 in WO 96/23874. When used as a maltose generating enzyme fungal alpha-amylases may be added in an amount of 0.001-1.0 AFAU/g DS, preferably from 0.002-0.5 AFAU/g DS, preferably 0.02-0.1 AFAU/g DS.

25 Preferably the alpha-amylase is an acid alpha-amylase, preferably from the genus *Aspergillus*, preferably of the species *Aspergillus niger*. In a preferred embodiment the acid fungal alpha-amylase is the one from *A. niger* disclosed as "AMYA\_ASPNG" in the Swiss-prot/TrEMBL database under the primary accession no. P56271. Also variants of set acid fungal amylase having at least 70% identity, such as at least 80% or even at least 90%, 95%, 96%, 97%, 98% or 99% identity thereto is contemplated. In an embodiment the acid fungal alpha-amylase is the one disclosed in SEQ ID NO: 1, or a sequence being at least 70% identical, preferably at least 75%, 80%, 85% or at least 90%, e.g. at least 95%, 97%, 98%, or at least 99% identity to SEQ ID NO:1.

35 Fungal acid alpha-amylase are preferably added in an amount of 0.001-10 AFAU/g of DS, in an amount of 0.01-0.25 AFAU/g of DS, or more preferably in an amount of 0.05-0.20 AFAU/kg of DS, such as around 0.1 AFAU/k DS.

### Commercial Alpha-amylases

Preferred commercial compositions comprising an alpha-amylase include MYCOLASE™ from DSM; BAN™, TERMAMYL™ SC, FUNGAMYL™, LIQUOZYME™ X and SAN™ SUPER, SAN™ EXTRA L from Novozymes A/S, Denmark) and CLARASE™ L-40,000, DEX-LO™, SPEYME FRED, SPEZYME™ ETHYL, SPEZYME™ AA, and SPEZYME™ DELTA AA (Genencor Int., USA), and the acid fungal alpha-amylase sold under the trade name SP 288 (available from Novozymes A/S, Denmark).

### **Carbohydrate-Source Generating Enzyme**

The term "carbohydrate-source generating enzyme" includes glucoamylase (being a glucose generator), beta-amylase and maltogenic amylases (being maltose generators). A carbohydrate-source generating enzyme is capable of providing energy to the fermenting organism(s) used in a process of the invention for producing the desired fermentation product, especially ethanol. The generated carbohydrate may be converted directly or indirectly to the desired fermentation product. The carbohydrate-source generating enzyme may be mixtures of enzymes falling within the definition. Especially contemplated mixtures are mixtures of at least a glucoamylase and an alpha-amylase, especially an acid amylase, even more preferred an acid fungal alpha-amylase. The ratio between acidic fungal alpha-amylase activity (AFAU) per glucoamylase activity (AGU) (AFAU per AGU) may in an embodiment of the invention be at least 0.1, in particular at least 0.16, such as in the range from 0.12 to 0.50.

Examples of contemplated glucoamylases, maltogenic amylases, and beta-amylases are set forth in the sections above and below.

### Glucoamylase

A glucoamylase used according to the invention may be derived from any suitable source, e.g., derived from a microorganism or a plant. Preferred glucoamylases are of fungal or bacterial origin, selected from the group consisting of *Aspergillus* glucoamylases, in particular *A. niger* G1 or G2 glucoamylase (Boel et al. (1984), EMBO J. 3 (5), p. 1097-1102), or variants thereof, such as disclosed in WO 92/00381, WO 00/04136 add WO 01/04273 (from Novozymes, Denmark); the *A. awamori* glucoamylase (WO 84/02921), *A. oryzae* (Agric. Biol. Chem. (1991), 55 (4), p. 941-949), or variants or fragments thereof.

Other *Aspergillus* glucoamylase variants include variants to enhance the thermal stability: G137A and G139A (Chen et al. (1996), Prot. Eng. 9, 499-505); D257E and D293E/Q (Chen et al. (1995), Prot. Engng. 8, 575-582); N182 (Chen et al. (1994), Biochem. J. 301, 275-281); disulphide bonds, A246C (Fierobe et al. (1996), Biochemistry, 35, 8698-8704; and

introduction of Pro residues in position A435 and S436 (Li et al. (1997), Protein Engng. 10, 1199-1204. Other glucoamylases include *Athelia rolfsii* (previously denoted *Corticium rolfsii*) glucoamylase (see US patent no. 4,727,026 and (Nagasaka, Y. et al. (1998) Purification and properties of the raw-starch-degrading glucoamylases from *Corticium rolfsii*, Appl Microbiol  
5 Biotechnol 50:323-330), *Talaromyces* glucoamylases, in particular, derived from *Talaromyces emersonii* (WO 99/28448), *Talaromyces leycettanus* (US patent no. Re. 32,153), *Talaromyces duponti*, *Talaromyces thermophilus* (US patent no. 4,587,215). Bacterial glucoamylases contemplated include glucoamylases from the genus *Clostridium*, in particular *C. thermoamylolyticum* (EP 135,138), and *C. thermohydrosulfuricum* (WO 86/01831).

10 Commercially available compositions comprising glucoamylase include AMG 200L; AMG 300 L; SAN™ SUPER, SAN™ EXTRA L, SPIRIZYME™ PLUS, SPIRIZYME™ FUEL, SPIRIZYME™ B4U and AMG™ E (from Novozymes A/S); OPTIDEX™ 300 (from Genencor Int.); AMIGASE™ and AMIGASE™ PLUS (from DSM); G-ZYME™ G900, G-ZYME™ and G990 ZR (from Genencor Int.).

15 Glucoamylases may in an embodiment be added in an amount of 0.02-20 AGU/g DS, preferably 0.1-10 AGU/g DS, such as 2 AGU/g DS.

#### Beta-amylase

At least according to the invention the a beta-amylase (E.C 3.2.1.2) is the name tradi-  
20 tionally given to exo-acting maltogenic amylases, which catalyze the hydrolysis of 1,4-alpha-glucosidic linkages in amylose, amylopectin and related glucose polymers. Maltose units are successively removed from the non-reducing chain ends in a step-wise manner until the molecule is degraded or, in the case of amylopectin, until a branch point is reached. The maltose released has the beta anomeric configuration, hence the name beta-amylase.

25 Beta-amylases have been isolated from various plants and microorganisms (W.M. Fogarty and C.T. Kelly, Progress in Industrial Microbiology, vol. 15, pp. 112-115, 1979). These beta-amylases are characterized by having optimum temperatures in the range from 40°C to 65°C and optimum pH in the range from 4.5 to 7. A commercially available beta-amylase from barley is NOVOZYM™ WBA from Novozymes A/S, Denmark and SPEZYME™ BBA  
30 1500 from Genencor Int., USA.

#### Maltogenic amylase

The amylase may also be a maltogenic alpha-amylase. A "maltogenic alpha-amylase" (glucan 1,4-alpha-maltohydrolase, E.C. 3.2.1.133) is able to hydrolyze amylose and amy-  
35 lopectin to maltose in the alpha-configuration. A maltogenic alpha-amylase from *Bacillus stearothermophilus* strain NCIB 11837 is commercially available from Novozymes A/S under

the tradename MALTOGENASE™. Maltogenic alpha-amylases are described in US Patent nos. 4,598,048, 4,604,355 and 6,162,628, which are hereby incorporated by reference.

The maltogenic amylase may in a preferred embodiment be added in an amount of 0.05- 5 mg total protein/gram DS or 0.05- 5 MANU/g DS.

5

### Production of Enzymes

The enzymes referenced herein may be derived or obtained from any suitable origin, including, bacterial, fungal, yeast or mammalian origin. The term "derived" or means in this context that the enzyme may have been isolated from an organism where it is present  
10 natively, i.e., the identity of the amino acid sequence of the enzyme are identical to a native enzyme. The term "derived" also means that the enzymes may have been produced recombinantly in a host organism, the recombinant produced enzyme having either an identity identical to a native enzyme or having a modified amino acid sequence, e.g., having one or more amino acids which are deleted, inserted and/or substituted, i.e., a recombinantly produced enzyme which is a mutant and/or a fragment of a native amino acid sequence or an  
15 enzyme produced by nucleic acid shuffling processes known in the art. Within the meaning of a native enzyme are included natural variants. Furthermore, the term "derived" includes enzymes produced synthetically by, e.g., peptide synthesis. The term "derived" also encompasses enzymes which have been modified e.g., by glycosylation, phosphorylation, or by  
20 other chemical modification, whether *in vivo* or *in vitro*. The term "obtained" in this context means that the enzyme has an amino acid sequence identical to a native enzyme. The term encompasses an enzyme that has been isolated from an organism where it is present natively, or one in which it has been expressed recombinantly in the same type of organism or another, or enzymes produced synthetically by, e.g., peptide synthesis. With respect to  
25 recombinantly produced enzymes the terms "obtained" and "derived" refers to the identity of the enzyme and not the identity of the host organism in which it is produced recombinantly.

The enzymes may also be purified. The term "purified" as used herein covers enzymes free from other components from the organism from which it is derived. The term "purified" also covers enzymes free from components from the native organism from which it is  
30 obtained. The enzymes may be purified, with only minor amounts of other proteins being present. The expression "other proteins" relate in particular to other enzymes. The term "purified" as used herein also refers to removal of other components, particularly other proteins and most particularly other enzymes present in the cell of origin of the enzyme of the invention. The enzyme may be "substantially pure," that is, free from other components from the  
35 organism in which it is produced, that is, for example, a host organism for recombinantly produced enzymes. In preferred embodiment, the enzymes are at least 75% (w/w) pure, more preferably at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least

97%, at least 98%, or at least 99% pure. In another preferred embodiment, the enzyme is 100% pure.

The enzymes used according to the present invention may be in any form suitable for use in the processes described herein, such as, e.g., in the form of a dry powder or granulate, a non-dusting granulate, a liquid, a stabilized liquid, or a protected enzyme. Granulates may be produced, e.g., as disclosed in US Patent Nos. 4,106,991 and US 4,661,452, and may optionally be coated by process known in the art. Liquid enzyme preparations may, for instance, be stabilized by adding stabilizers such as a sugar, a sugar alcohol or another polyol, lactic acid or another organic acid according to established process. Protected enzymes may be prepared according to the process disclosed in EP 238,216.

Even if not specifically mentioned in context of a method or process of the invention, it is to be understood that the enzyme(s) or agent(s) is(are) used in an "effective amount".

## 15 MATERIALS AND METHODS

### Enzymes:

Bacterial Alpha-amylase A: *Bacillus stearothermophilus* alpha-amylase variant with the mutations: I181\*+G182\*+N193F disclosed in US patent no. 6,187,576 and available on request from Novozymes A/S, Denmark.

Fungal acid alpha-amylase B: *Aspergillus niger* alpha-amylase disclosed in SEQ ID NO: 1 and available from Novozymes A/S.

Glucoamylase T: Glucoamylase derived from *Talaromyces emersonii* and disclosed as SEQ ID NO: 7 in WO 99/28448.

Glucoamylase SF: Balanced blend of *Aspergillus niger* glucoamylase and *A. niger* acid alpha-amylase having a ratio between AGU and AFAU of approx. 9:1.

### 30 Stock solution for iodine method:

0.1N I<sub>2</sub>

· dissolve 1.3 g I<sub>2</sub> and 2.0 g KI into 100 mL DI water

### Methods:

#### 35 Alpha-amylase activity (KNU)

The amylolytic activity may be determined using potato starch as substrate. This method is based on the break-down of modified potato starch by the enzyme, and the reaction is followed by mixing samples of the starch/enzyme solution with an iodine solution. Ini-

tially, a blackish-blue color is formed, but during the break-down of the starch the blue color gets weaker and gradually turns into a reddish-brown, which is compared to a colored glass standard.

One Kilo Novo alpha amylase Unit (KNU) is defined as the amount of enzyme which, under standard conditions (i.e., at 37°C +/- 0.05; 0.0003 M Ca<sup>2+</sup>; and pH 5.6) dextrinizes 5260 mg starch dry substance Merck Amylum soluble.

A folder EB-SM-0009.02/01 describing this analytical method in more detail is available upon request to Novozymes A/S, Denmark, which folder is hereby included by reference.

10

#### Determination of FAU activity

One Fungal Alpha-Amylase Unit (FAU) is defined as the amount of enzyme, which breaks down 5.26 g starch (Merck Amylum soluble Erg. B.6, Batch 9947275) per hour based upon the following standard conditions:

15      Substrate . . . . . Soluble starch  
           Temperature . . . . . 37°C  
           pH. . . . . 4.7  
           Reaction time . . . . 7-20 minutes

#### 20      Determination of acid alpha-amylase activity (AFAU)

Acid alpha-amylase activity is measured in AFAU (Acid Fungal Alpha-amylase Units), which are determined relative to an enzyme standard.

The standard used is AMG 300 L (from Novozymes A/S, Denmark, glucoamylase wild-type *Aspergillus niger* G1, also disclosed in Boel et al. (1984), EMBO J. 3 (5), p. 1097-1102) and WO 92/00381). The neutral alpha-amylase in this AMG falls after storage at room temperature for 3 weeks from approx. 1 FAU/mL to below 0.05 FAU/mL.

The acid alpha-amylase activity in this AMG standard is determined in accordance with the following description. In this method, 1 AFAU is defined as the amount of enzyme, which degrades 5.260 mg starch dry matter per hour under standard conditions.

30      Iodine forms a blue complex with starch but not with its degradation products. The intensity of color is therefore directly proportional to the concentration of starch. Amylase activity is determined using reverse colorimetry as a reduction in the concentration of starch under specified analytic conditions.

	Alpha-amylase	
Starch + Iodine	→	Dextrins + Oligosaccharides
	40°C, pH 2.5	
Blue/violet	t=23 sec.	Decoloration

Standard conditions/reaction conditions: (per minute)

Substrate:	Starch, approx. 0.17 g/L
Buffer:	Citrate, approx. 0.03 M
Iodine (I <sub>2</sub> ):	0.03 g/L
CaCl <sub>2</sub> :	1.85 mM
pH:	2.50 ± 0.05
Incubation temperature:	40°C
Reaction time:	23 seconds
Wavelength:	lambda=590nm
Enzyme concentration:	0.025 AFAU/mL
Enzyme working range:	0.01-0.04 AFAU/mL

If further details are preferred these can be found in EB-SM-0259.02/01 available on request from Novozymes A/S, Denmark, and incorporated by reference.

#### Acid Alpha-amylase Units (AAU)

5           The acid alpha-amylase activity can be measured in AAU (Acid Alpha-amylase Units), which is an absolute method. One Acid Amylase Unit (AAU) is the quantity of enzyme converting 1 g of starch (100% of dry matter) per hour under standardized conditions into a product having a transmission at 620 nm after reaction with an iodine solution of known strength equal to the one of a color reference.

Standard conditions/reaction conditions:

Substrate:	Soluble starch. Concentration approx. 20 g DS/L.
Buffer:	Citrate, approx. 0.13 M, pH=4.2
Iodine solution:	40.176 g potassium iodide + 0.088 g iodine/L
City water	15°-20°dH (German degree hardness)
pH:	4.2
Incubation temperature:	30°C
Reaction time:	11 minutes
Wavelength:	620nm
Enzyme concentration:	0.13-0.19 AAU/mL
Enzyme working range:	0.13-0.19 AAU/mL

10           The starch should be Lintner starch, which is a thin-boiling starch used in the laboratory as colorimetric indicator. Lintner starch is obtained by dilute hydrochloric acid treatment of native starch so that it retains the ability to color blue with iodine. Further details can be found in EP0140410B2, which disclosure is hereby included by reference.

#### 15   Glucoamylase activity (AGI)

Glucoamylase (equivalent to amyloglucosidase) converts starch into glucose. The amount of glucose is determined here by the glucose oxidase method for the activity determination. The method described in the section 76-11 Starch—Glucoamylase Method with Subsequent Measurement of Glucose with Glucose Oxidase in "Approved methods of the American Association of Cereal Chemists". Vol.1-2 AACC, from American Association of Cereal Chemists, (2000); ISBN: 1-891127-12-8.

One glucoamylase unit (AGI) is the quantity of enzyme which will form 1 micromol of glucose per minute under the standard conditions of the method.

Standard conditions/reaction conditions:

10

Substrate:	Soluble starch. Concentration approx. 16 g dry matter/L.
Buffer:	Acetate, approx. 0.04 M, pH=4.3
pH:	4.3
Incubation temperature:	60°C
Reaction time:	15 minutes
Termination of the reaction:	NaOH to a concentration of approximately 0.2 g/L (pH~9)
Enzyme concentration:	0.15-0.55 AAU/mL

The starch should be Lintner starch, which is a thin-boiling starch used in the laboratory as colorimetric indicator. Lintner starch is obtained by dilute hydrochloric acid treatment of native starch so that it retains the ability to color blue with iodine.

#### 15 Glucoamylase activity (AGU)

The Novo Glucoamylase Unit (AGU) is defined as the amount of enzyme, which hydrolyzes 1 micromole maltose per minute under the standard conditions 37°C, pH 4.3, substrate: maltose 23.2 mM, buffer: acetate 0.1 M, reaction time 5 minutes.

20 An autoanalyzer system may be used. Mutarotase is added to the glucose dehydrogenase reagent so that any alpha-D-glucose present is turned into beta-D-glucose. Glucose dehydrogenase reacts specifically with beta-D-glucose in the reaction mentioned above, forming NADH which is determined using a photometer at 340 nm as a measure of the original glucose concentration.

AMG incubation:	
Substrate:	maltose 23.2 mM
Buffer:	acetate 0.1 M
pH:	4.30 ± 0.05
Incubation temperature:	37°C ± 1

Reaction time:	5 minutes
Enzyme working range:	0.5-4.0 AGU/mL

Color reaction:	
GlucDH:	430 U/L
Mutarotase:	9 U/L
NAD:	0.21 mM
Buffer:	phosphate 0.12 M; 0.15 M NaCl
pH:	7.60 $\pm$ 0.05
Incubation temperature:	37°C $\pm$ 1
Reaction time:	5 minutes
Wavelength:	340 nm

A folder (EB-SM-0131.02/01) describing this analytical method in more detail is available on request from Novozymes A/S, Denmark, which folder is hereby included by reference.

5

#### Determination of Maltogenic Amylase activity (MANU)

One MANU (Maltogenic Amylase Novo Unit) may be defined as the amount of enzyme required to release one micro mole of maltose per minute at a concentration of 10 mg of maltotriose (Sigma M 8378) substrate per ml of 0.1 M citrate buffer, pH 5.0 at 37°C for 30 minutes.

10

#### Standard iodine method

- boil small aliquot (10-20 mLs) of liquefied material in a test tube for several minutes
- cool in ice bath
- add 10-12 drops of the iodine solution
- mix and let sample sit in ice water for about 10 minutes

15

#### Determination of Viscosity

The mash is heated to a temperature of 50-70°C, depending on the treatment. Following treatment viscosity is measured using a Haake VT02 rotation based viscosimeter. The unit of viscosity is centipois (cps), which is proportionally related to the viscosity level.

20

#### Determination of DE (Dextrose Equivalent)

The DE value is measured using Fehlings liquid by forming a copper complex with the starch using pure glucose as a reference, which subsequently is quantified through io-

25

dometric titration. DE (dextrose equivalent) is defined as the amount of reducing carbohydrate (measured as dextrose-equivalents) in a sample expressed as w/w% of the total amount of dissolved dry matter. It may also be measured by the neocuproine assay (Dygert, Li Floridana(1965) Anal. Biochem. No 368). The principle of the neocuproine assay is that

5 CuSO<sub>4</sub> is added to the sample, Cu<sup>2+</sup> is reduced by the reducing sugar and the formed neocuproine complex is measured at 450 nm.

#### Degree of Identity

The degree of identity between two amino acid sequences is determined by the

10 Clustal method (Higgins, 1989, CABIOS 5: 151-153) using the LASERGENE™ MEGALIGN™ software (DNASTAR, Inc., Madison, WI) with an identity table and the following multiple alignment parameters: Gap penalty of 10, and gap length penalty of 10. Pair-wise alignment parameters were Ktuple=1, gap penalty=3, windows=5, and diagonals=5].

15

### **EXAMPLES**

#### **Example 1**

##### Liquefaction with bacterial and acid alpha amylase

20 400 mL of ground corn slurry is liquefied with 50 NU/g dry solids (DS) Bacterial Alpha-Amylase A. The corn mash has about 30% dry substance (pH 5.4). The mash is heated to 85°C and the viscosity and DE values are measured.

The mash is then treated with acid alpha-amylase B from *Aspergillus niger* having the amino acid sequence disclosed in SEQ ID NO:1. The enzyme loading is 0.10 AFAU/g dry

25 solids. After 1.5 hours the viscosity and DE value are measured.

#### **Example 2**

##### The Effect of Alpha-Amylase Addition During Liquefaction on SSF Performance:

To investigate the effect of alpha-amylase (in form of Acid Alpha-Amylase B) addition in liquefaction, six different conditions for liquefaction were tested. To begin with ground

30 corn was used to make 30% slurry with tap water. The pH in all the liquefactions were adjusted to 5.4 using diluted H<sub>2</sub>SO<sub>4</sub>. In the first two liquefactions (controls), Bacterial Alpha-Amylase A (50 NU/ g DS) was added and kept at 85°C for 1.5 and 4.5 hours, respectively. In the second set of runs (Acid Alpha-Amylase B test), the same process was followed, but in-

35 cubation time with Bacterial Alpha-Amylase A at 85 °C was reduced to 0.5 hours, the temperature was then lowered and Acid Alpha-Amylase B was added (0.050 and 0.10 AFAU/g

DS). The mixture was then kept at 70°C for 1 and 4 hours, respectively. Once the liquefaction was over, the reactions were stopped by adding 2 drops of HCl (4 N). Samples were withdrawn to analyze sugar profiles (using HPLC) and DE values. The liquefied samples were frozen and later subjected to SSF.

- 5 The effect of liquefaction treatment on SSF was evaluated via mini-scale fermentations. Samples after liquefaction were thawed and the pH was adjusted to 5.0 with diluted H<sub>2</sub>SO<sub>4</sub>. Approximately 4 grams of mash was added to 16 ml polystyrene tubes (Falcon 352025). Tubes were then dosed with the appropriate amount of Glucoamylase SF (0.3 AGU/g DS). Six replicates of each treatment were run. After dosing the tubes with enzyme, 10 they were inoculated with 0.04 ml/g mash of yeast propagate that had been grown for 21 hours on corn mash. Vials were capped with a screw on lid which had been punctured with a very small needle to allow gas release and vortexed briefly before weighing and incubation at 32°C. Fermentation progress was followed by weighing the tubes over time. Tubes were vortexed briefly before each weighing. Weight loss values were converted to ethanol yield (g 15 ethanol/g DS) (see Fig. 1) by the following formula:

$$g_{ethanol/g DS} = \frac{g_{CO_2} \text{ weight loss} \times \frac{1 \text{ mol } CO_2}{440098 \text{ g } CO_2} \times \frac{1 \text{ mole ethanol}}{1 \text{ mol } CO_2} \times \frac{46094 \text{ g ethanol}}{1 \text{ mole ethanol}}}{g_{corn \text{ in tube}} \times \%DS \text{ of corn}}$$

- 20 Data from the liquefaction process shows that adding Acid Alpha-Amylase B in addition to Bacterial alpha-amylase A resulted in a significant increase in DE values.

Treatment	enzyme1	enzyme 2	DE
1	BAA(50)-85°C-1.5hr		7.27
2	BAA(50)-85°C-4.5hr		7.79
3	BAA(50)-85°C-0.5hr	AAA(50)-70°C-1hr	16.24
4	BAA(50)-85°C-0.5hr	AAA(50)-70°C-4hr	20.79
5	BAA(50)-85°C-0.5hr	AAA(100)-70°C-1hr	22.13
6	BAA(50)-85°C-0.5hr	AAA(100)-70°C-4hr	23.56

\* BAA: Bacterial alpha-amylase

\* AAA: Acid alpha-amylase

**CLAIMS**

1. A method of liquefying starch-containing material, wherein the method comprises the steps of
  - 5 (a) treating the starch-containing material with a bacterial alpha-amylase at a temperature around 70-90°C for 15-90 minutes,
  - (b) treating the material obtained in step (a) with an alpha-amylase at a temperature between 60-80°C for 30-90 minutes.
- 10 2. The method of claim 1, wherein the starch-containing material is jet-cooking at 90-120°C, preferably around 105°C, for 1-15 minutes, preferably for 3-10 minute, especially around 5 minutes, before step (a).
3. The method of claims 1 or 2, wherein the starch-containing material is selected from the
  - 15 group consisting of: tubers, roots and whole grain; and any combinations of these.
4. The method of any of claims 1-5, wherein the starch-containing material is obtained from cereals.
- 20 5. The method of any of the claims 1-5, wherein the starch-containing material is selected from the group consisting of corn, cob, wheat, barley, rye, milo and potatoes; or any combination of these.
6. The method of claim 5, wherein the starch-containing material is whole grain selected
  - 25 from the group consisting of corn, wheat or barley or any combinations of these.
7. The method of any of the claims 1-8, wherein the starch-containing material is whole grain and said method comprises a step of milling the whole grain before step (a).
- 30 8. The method of any of the claims 1-5, wherein the starch-containing material is obtainable by a process comprising milling of whole grain.
9. The method of any of the claims 1-8, further comprising prior to step (a) the steps of;
  - i) milling of starch-containing material;
  - 35 ii) forming a slurry comprising the milled material and water.

10. The method of any of the claim 9, wherein the milling step is a dry milling step.
11. The method of any of the claims 9-10, wherein the milling step is a wet milling step.
- 5 12. The method of any of the claims 1-11, wherein the starch-containing material is a side stream from starch processing.
- 10 13. The method of claims 1-12, wherein the bacterial alpha-amylase in step (a) is a *Bacillus* alpha-amylase, preferably derived from *Bacillus stearothermophilus* alpha-amylase or a variant with the mutations: I181\*+G182\* especially I181\*+G182\*+N193F.
- 15 14. The method of claims 1-13 wherein the alpha-amylase in step (b) is an acid alpha-amylase, preferably an acid fungal alpha-amylase, preferably derived from *Aspergillus* spp. preferably *Aspergillus niger* or *Aspergillus oryzae*.
- 20 15. The method of any of claims 1-14, wherein the acid alpha-amylase is an alpha-amylase having an amino acid sequence which has at least 70% identity to SEQ ID NO:1 preferably at least 75%, 80%, 85% or at least 90%, e.g., at least 95%, 97%, 98%, or at least 99% identity to SEQ ID NO:1.
16. The method of claims 1-15, wherein the acid alpha-amylase is an alpha-amylase having the amino acid sequence set forth in SEQ ID NO:1.
- 25 17. The method of claims 1-16, wherein the mash obtained after step (b) has a DE value of above 16, preferably above 18, especially above 20, such as a DE value in the range from 16 to 30, preferably in the range from 18 to 25.
- 30 18. A process of producing a fermentation product from starch-containing material by fermentation, said process comprises:
- (i) liquefying said starch-containing material as defined in any of claims 1 to 17;
  - (ii) saccharifying the liquefied mash obtained;
  - (iii) fermenting.
- 35 19. The process of claim 18, further comprising recovery of the fermentation product.

20. The process of claims 18 or 19, wherein the fermentation product is ethanol.
21. The process of any of claims 18 to 20, wherein the saccharification and fermentation is carried out as a simultaneous saccharification and fermentation process (SSF process).
- 5 22. The process of any of claims 18 to 21, further comprising a step of;  
(iv) distillation to obtain ethanol;  
wherein the fermentation in step (iii) and the distillation in step (iv) is carried out simultaneously or separately/sequentially; optionally followed by one or more process steps for further  
10 refinement of ethanol.
23. The process of claim 18, wherein a fermenting organism capable of fermenting sugars to ethanol is used in step iii).
- 15 24. The process of any of claims 18 to 23, wherein the fermenting organism in step iii) is yeast, such as a strain of *Saccharomyces spp.*, preferably a strain of *Saccharomyces cerevisiae*.
25. The process of any of the claims 18 to 24, wherein the fermentation is carried out in  
20 the presence of a carbohydrate-source generating enzyme.
26. The process of claim 25, wherein the carbohydrate-source generating enzyme is glucoamylase, preferably derived from a strain of *Aspergillus*, preferably *Aspergillus niger* or *Aspergillus awamori*, a strain of *Talaromyces*, especially *Talaromyces emersonii*, or a strain  
25 of *Athelia*, especially *Athelia rolfsii*
27. The process according to any of claims 18 to 26, said process comprising the steps of;  
1) liquefying starch-containing material in accordance with the liquefaction method of claims 1-17;  
30 2) liquefying the material obtained in step 1) in the presence of an alpha-amylase having an amino acid sequence which has at least 70% identity to SEQ ID NO:1; and  
3) saccharifying the material obtained in step 2) ;  
4) fermenting;  
wherein the steps 1), 2), 3) and 4) is performed in the order 1), 2), 3), 4) or wherein 4) is  
35 performed simultaneously to or following 3).

28. The process of claim 27, wherein step 4) is carried out using a fermenting organism capable of fermenting sugars to ethanol.

29. The process of claim 28, wherein the fermenting organism is yeast, preferably a strain of  
5 *Saccharomyces spp.*, preferably a strain of *Saccharomyces cerevisiae*.

30. The method of any of claims 27 to 29, wherein the mash obtained in step 2) has a DE value of above 16, preferably above 18, especially above 20, such as a DE value in the range from 16 to 30, preferably in the range from 18 to 25.  
10

31. A process of producing syrup from starch-containing material, comprising  
    (a) liquefying starch-containing material in accordance with the liquefaction method of any of claims 1 to 17,  
    (b) saccharifying the liquefied material.  
15

32. The process of claim 31, wherein the syrup is selected from the group comprising glucose, maltose, fructose syrups, malto-oligosaccharides, and isomalto-oligosaccharides.  
20

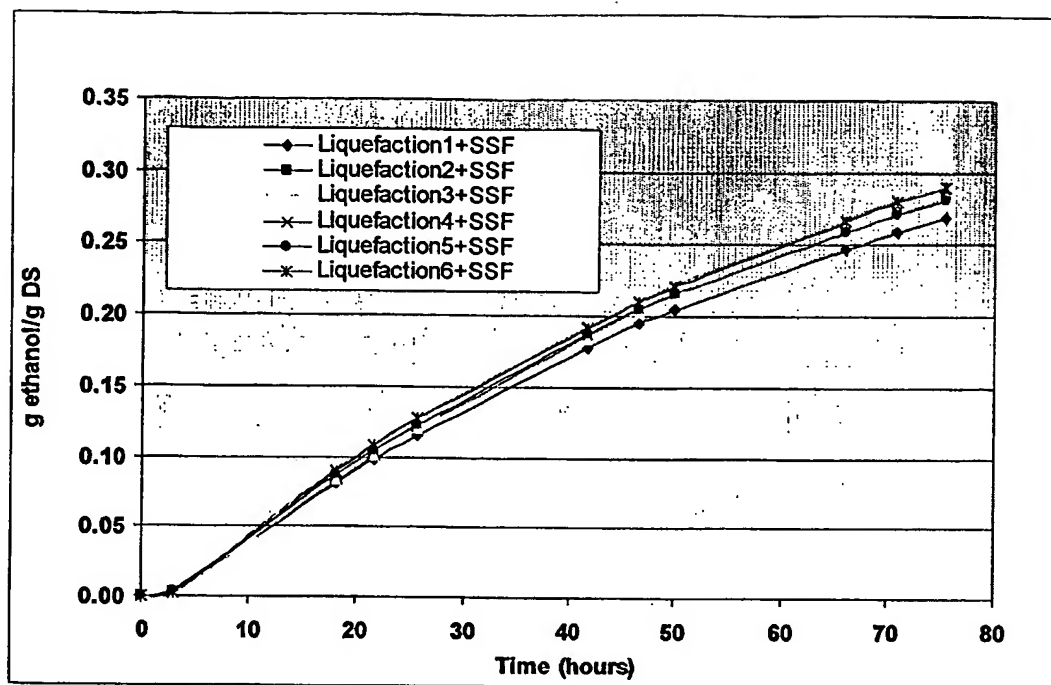


Fig. 1

## SEQUENCE LISTING

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<210> 1

<211> 484

<212> PRT

<213> Aspergillus niger

<220>

<221> misc\_feature

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Ile Thr Glu Gln Leu Pro Gln Asp Thr Ala Asp Gly Glu Ala Tyr His  
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Gly Tyr Trp Gln Gln Lys Ile Tyr Asp Val Asn Ser Asn Phe Gly Thr  
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Tyr Leu Met Val Asp Val Val Pro Asp His Met Gly Tyr Ala Gly Asn  
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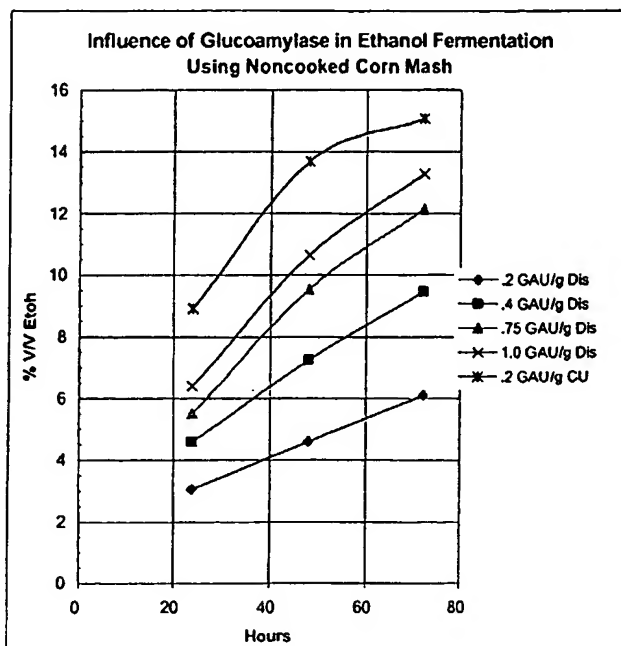
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- (54) Title: **METHODS FOR PRODUCING ETHANOL FROM CARBON SUBSTRATES**



(57) Abstract: The present invention provides means for the production of desired end-products of in vitro and/or in vivo bioconversion of biomass-based feed stock substrates, including but not limited to such materials as starch and cellulose. In particularly preferred embodiments, the methods of the present invention do not require gelatinization and/or liquefaction of the substrate. In particularly preferred embodiments, the present invention provides means for the production of ethanol.

# **METHODS FOR PRODUCING ETHANOL FROM CARBON SUBSTRATES**

## **FIELD OF INVENTION**

The present invention provides means for the production of desired end-products of *in vitro* and/or *in vivo* bioconversion of biomass-based feed stock substrates, including but not limited to such materials as starch and cellulose. In particularly preferred embodiments, the methods of the present invention do not require gelatinization and/or liquefaction of the substrate. In particularly preferred embodiments, the present invention provides means for the production of ethanol.

## **BACKGROUND OF THE INVENTION**

Industrial fermentations predominantly utilize glucose as feed-stock for the production of proteins, enzymes and chemicals. These fermentations are usually batch, fed-batch, or continuous, and operate under conditions that are substrate-limited and/or designed to produce minimal by-products. As those in the art know, there are certain critical operating conditions that must be controlled during fermentation so as to optimize fermentation time, yield and efficiency.

Glucose is a natural, carbon based compound that is useful in a multitude of chemical and biological synthetic applications as a starting substrate. However, syrups that contain glucose purity levels of greater than 90% are relatively expensive. In addition, the presence of high glucose concentrations increases the susceptibility of the fermentation system to microbial contamination, thereby resulting in an adverse effect upon the production efficiency. Another disadvantage is that even the presence of low to moderate levels of glucose in the fermentation vat adversely affects the conversion of the glucose to the desired end product, for example by enzymatic inhibition and/or catabolite repression, and/or the growth of microorganisms. As a result, various attempts have been made to reduce

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the costs of industrial fermentation, particularly in utilization of substrates that are less expensive than glucose. However, despite the development of numerous approaches, there remains a need in the art for economical, efficiently-utilized substrates for fermentation. Indeed, there is a great need in the art for methods  
5 that utilize a less expensive starting material than glucose to more efficiently produce a desired end-product.

### SUMMARY OF THE INVENTION

The present invention provides means for the production of desired end-  
10 products of *in vitro* and/or *in vivo* bioconversion of biomass-based feed stock substrates, including but not limited to such materials as starch and cellulose. In particularly preferred embodiments, the methods of the present invention do not require gelatinization and/or liquefaction of the substrate. In particularly preferred embodiments, the present invention provides means for the production of ethanol.  
15 In some particularly preferred embodiments, the present invention provides means for the production of ethanol directly from granular starch, in which altered catabolite repression is involved.

In some embodiments, the present invention provides methods for producing ethanol in which the glucose concentration of the conversion medium is maintained  
20 at a low concentration, preferably below the threshold triggering catabolite repression and/or enzyme inhibition, so as to increase efficiency of the process by avoiding catabolic repressive and/or enzymatic inhibitive effects of glucose upon the enzymatic conversion of starch to ethanol.

In additional embodiments, the present invention provides methods for  
25 producing ethanol comprising the steps of contacting at least one carbon substrate with at least one substrate converting enzyme, to produce at least one intermediate, and then contacting at least one intermediate with at least one intermediate producing enzyme in a reactor vessel, wherein the at least one intermediate is substantially all bioconverted an end-product. In some preferred embodiments, a  
30 microorganism is used to achieve this bioconversion. By maintaining a low concentration of the intermediate in a conversion medium, the intermediate's catabolite repressive and/or enzymatic inhibitive effects are altered (e.g., reduced).

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The present invention also provides various levels of intermediate concentration, substrates, intermediates and steps of converting the intermediate to ethanol.

The present invention provides methods for producing an alcohol as an end-product comprising the steps of: contacting a carbon substrate and at least one  
5 substrate-converting enzyme to produce an intermediate; and contacting the intermediate with at least one intermediate-converting enzyme, wherein the intermediate is substantially all converted by the intermediate enzyme to alcohol. In some preferred embodiments, the intermediate-converting enzyme is a microbial enzyme. In alternative preferred embodiments, the intermediate-converting  
10 microbial enzyme is secreted by a microorganism that is in contact with the intermediate. In further embodiments, substrate-converting enzyme is a microbial enzyme. In some preferred embodiments, the substrate-converting microbial enzyme is secreted by a microorganism that is in contact with the substrate. In still other preferred embodiments, the intermediate-converting enzyme and the  
15 substrate-converting enzyme are produced by microorganisms of the same species. In alternative embodiments, the intermediate-converting enzyme and the substrate-converting enzyme are produced by microorganisms of the different species. In still further embodiments, the concentration level of the intermediate is maintained at a level below that which triggers catabolite repression effects upon the conversion of  
20 the intermediate to the end-product. In additional embodiments, concentration level of the intermediate is maintained at a level below that which triggers enzymatic inhibition effects upon the conversion of the intermediate to the end-product. In some preferred embodiments, the intermediate is converted to the end-product at a rate sufficient to maintain the concentration that is less than 0.25%. In yet other  
25 embodiments, the substrate is selected from the group consisting of biomass and starch. In some preferred embodiments, the biomass comprises corn solids. In some particularly preferred embodiments, intermediate is selected from the group consisting of hexoses and pentoses. In some embodiments, the hexose is glucose. In some embodiments, the substrate is cooked prior to its use in the present  
30 invention, while in other embodiments, the substrate is uncooked prior to its use in the present invention. In yet other embodiments, the step of contacting the substrate and substrate-converting enzyme further comprises bioconverting the

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substrate to produce the intermediate. In most preferred embodiments, the alcohol end-product is ethanol. In still further embodiments, the step of contacting the substrate and at least one substrate-converting enzyme further comprises providing an amount of the substrate-converting enzyme at a concentration that produces the intermediate at a concentration that is less than or equal to the amount of the intermediate converted by at least one intermediate-converting enzyme. In some additional embodiments, at least one substrate-converting enzyme converts at least 50% of the substrate to the intermediate within 72 hours, while in other embodiments, at least one substrate-converting enzyme converts at least 90% of the substrate to the intermediate within 72 hours, and in some preferred embodiments, at least one substrate-converting enzyme converts at least 95% of the substrate to the intermediate within 72 hours. In still further embodiments, at least one substrate-converting enzyme and at least one intermediate-converting enzyme are obtained from a microorganism selected from the group consisting of *Rhizopus* and *Aspergillus*. In additional embodiments, the substrate-converting and/or intermediate-converting enzyme(s) are provided as a cell-free extract. In further preferred embodiments, the contacting steps take place in a reaction vessel, including but not limited to vats, bottles, flasks, bags, bioreactors, and any other receptacle suitable for conducting the methods of the present invention.

The present invention further provides methods for producing alcohol as an end-product comprising the steps of contacting a carbon substrate and at least one substrate-converting enzyme to produce an intermediate; and contacting the intermediate with at least one intermediate-converting enzyme, wherein the intermediate is substantially all converted by the intermediate enzyme to the alcohol end-product, and wherein the presence of the end-product does not inhibit the further production of the alcohol end-product. In some preferred embodiments, the intermediate-converting enzyme is a microbial enzyme. In alternative preferred embodiments, the intermediate-converting microbial enzyme is secreted by a microorganism that is in contact with the intermediate. In further preferred embodiments, the substrate-converting enzyme is a microbial enzyme. In still further embodiments, the substrate-converting microbial enzyme is secreted by a microorganism that is in contact with the substrate. In some embodiments,

- 5 -

intermediate-converting enzyme and the substrate-converting enzyme are produced by microorganisms of the same species, while in other embodiments, intermediate-converting enzyme and the substrate-converting enzyme are produced by microorganisms of the different species. In yet other embodiments, the substrate is  
5 selected from the group consisting of biomass and starch. In some preferred embodiments, the biomass comprises corn solids. In some particularly preferred embodiments, intermediate is selected from the group consisting of hexoses and pentoses. In some embodiments, the hexose is glucose. In some embodiments, the substrate is cooked prior to its use in the present invention, while in other  
10 embodiments, the substrate is uncooked prior to its use in the present invention. In some particularly preferred embodiments, the alcohol end-product is ethanol. In still further embodiments, the step of contacting the substrate and at least one substrate-converting enzyme further comprises providing an amount of the substrate-converting enzyme at a concentration that produces the intermediate at a  
15 concentration that is less than or equal to the amount of the intermediate converted by at least one intermediate-converting enzyme. In some additional embodiments, at least one substrate-converting enzyme converts at least 50% of the substrate to the intermediate within 72 hours, while in other embodiments, at least one substrate-converting enzyme converts at least 90% of the substrate to the  
20 intermediate within 72 hours, and in some preferred embodiments, at least one substrate-converting enzyme converts at least 95% of the substrate to the intermediate within 72 hours. In still further embodiments, at least one substrate-converting enzyme and at least one intermediate-converting enzyme are obtained from a microorganism selected from the group consisting of *Rhizopus* and  
25 *Aspergillus*. In additional embodiments, the substrate-converting and/or intermediate-converting enzyme(s) are provided as a cell-free extract. In further preferred embodiments, the contacting steps take place in a reaction vessel, including but not limited to vats, bottles, flasks, bags, bioreactors, and any other receptacle suitable for conducting the methods of the present invention.

30 The present invention further provides methods for producing an alcohol end-product comprising the steps of: contacting a carbon substrate and at least one substrate-converting enzyme to produce an intermediate; and contacting the

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intermediate with at least one intermediate-converting enzyme, wherein the intermediate is substantially all converted by the intermediate enzyme to the alcohol end-product, and wherein the presence of the substrate does not inhibit the further production of the alcohol end-product. In some preferred embodiments, the  
5 intermediate-converting enzyme is a microbial enzyme. In alternative preferred embodiments, the intermediate-converting microbial enzyme is secreted by a microorganism that is in contact with the intermediate. In further preferred embodiments, the substrate-converting enzyme is a microbial enzyme. In still further embodiments, the substrate-converting microbial enzyme is secreted by a  
10 microorganism that is in contact with the substrate. In some embodiments, intermediate-converting enzyme and the substrate-converting enzyme are produced by microorganisms of the same species, while in other embodiments, intermediate-converting enzyme and the substrate-converting enzyme are produced by microorganisms of the different species. In yet other embodiments, the substrate is  
15 selected from the group consisting of biomass and starch. In some preferred embodiments, the biomass comprises corn solids. In some particularly preferred embodiments, intermediate is selected from the group consisting of hexoses and pentoses. In some embodiments, the hexose is glucose. In some embodiments, the substrate is cooked prior to its use in the present invention, while in other  
20 embodiments, the substrate is uncooked prior to its use in the present invention. In some particularly preferred embodiments, the alcohol end-product is ethanol. In still further embodiments, the step of contacting the substrate and at least one substrate-converting enzyme further comprises providing an amount of the substrate-converting enzyme at a concentration that produces the intermediate at a  
25 concentration that is less than or equal to the amount of the intermediate converted by at least one intermediate-converting enzyme. In some additional embodiments, at least one substrate-converting enzyme converts at least 50% of the substrate to the intermediate within 72 hours, while in other embodiments, at least one substrate-converting enzyme converts at least 90% of the substrate to the  
30 intermediate within 72 hours, and in some preferred embodiments, at least one substrate-converting enzyme converts at least 95% of the substrate to the intermediate within 72 hours. In still further embodiments, at least one substrate-

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converting enzyme and at least one intermediate-converting enzyme are obtained from a microorganism selected from the group consisting of *Rhizopus* and *Aspergillus*. In additional embodiments, the substrate-converting and/or intermediate-converting enzyme(s) are provided as a cell-free extract. In further preferred embodiments, the contacting steps take place in a reaction vessel, including but not limited to vats, bottles, flasks, bags, bioreactors, and any other receptacle suitable for conducting the methods of the present invention.

#### BRIEF DESCRIPTION OF THE FIGURES

Figure 1 provides a graph showing the ethanol results for the experiments described in Example 1.

Figure 2, Panels A, B and C provide graphs showing the ethanol results from uncooked ground corn fermentation using M1 (Panel A), CU (Panel B), and M1 with DISTILLASE® (Panel C).

Figure 3, Panels A, B and C provide graphs showing the ethanol results obtained in the experiments described in Example 3.

Figure 4 shows the response of ethanol to the amount of stillage added in both types of mashes.

Figure 5 shows the glucose profile after 72 hour of fermentation as described in Example 4.

Figure 6 is a plot of the disaccharides after 72 hours of fermentation with respect to stillage added (See, Example 4).

Figure 7 shows the levels of the higher sugars (*i.e.*, oligosaccharides greater than disaccharides) (See, Example 4).

Figure 8 shows the lactic acid level after 72 hours of fermentation (See, Example 4).

Figure 9 summarizes the glycerol levels after 72 hours of fermentation (See, Example 4).

#### DESCRIPTION OF THE INVENTION

The present invention provides means for the production of desired end-products of *in vitro* and/or *in vivo* bioconversion of biomass-based feed stock

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substrates, including but not limited to such materials as starch and cellulose. In particularly preferred embodiments, the methods of the present invention do not require gelatinization and/or liquefaction of the substrate. In particularly preferred embodiments, the present invention provides means for the production of ethanol.

5 In some particularly preferred embodiments, the present invention provides means for the production of ethanol directly from granular starch, in which altered catabolite repression is involved.

In particular, the present invention provides means for making ethanol in a manner that is characterized by having altered levels of catabolite repression and enzymatic inhibition, thus increasing the process efficiency. The methods of the present invention comprise the steps of contacting a carbon substrate and a substrate converting enzyme to produce an intermediate; and contacting the intermediate with an intermediate producing enzyme in a reactor vessel, wherein the intermediate is substantially all bioconverted by an end-product producing

10 microorganism. By maintaining a low concentration of the intermediate in a conversion medium, the catabolite repressive or enzymatic inhibitive effects of the intermediate on the process are altered.

The present invention also provides methods in which starches or biomass and hydrolyzing enzymes are used to convert starch or cellulose to glucose. In addition, the present invention provides methods in which these substrates are

20 provided at such a rate that the conversion of starch to glucose matches the glucose feed rate required for the respective fermentative product formation. Thus, the present invention provides key glucose-limited fermentative conditions, as well as avoiding many of the metabolic regulations and inhibitions.

In some preferred embodiments, the present invention provides means for making desired end-products, in which a continuous supply of glucose is provided under controlled rate conditions, providing such benefits as reduced raw material cost, lower viscosity, improved oxygen transfer for metabolic efficiency, improved bioconversion efficiency, higher yields, altered levels of catabolite repression and enzymatic inhibition, and lowered overall manufacturing costs.

25 30

As indicated above, there is a great need in the art for methods in which less expensive starting materials than glucose are used to efficiently produce a desired

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end-product. As described in greater detail herein, the present invention provides methods involving such substrates, including starch (e.g., corn and wheat starch) and biomass.

Starch is a plant-based fermentation carbon source. Corn starch and wheat  
5 starch are carbon sources that are much cheaper than glucose carbon feedstock for fermentation. Conversion of liquefied starch to glucose is known in the art and is generally carried out using enzymes such alpha-amylase, pullulanase, and glucoamylase. A large number of processes have been described for converting liquefied starch to the monosaccharide, glucose. Glucose has value in itself, and  
10 also as a precursor for other saccharides such as fructose. In addition, glucose may also be fermented to ethanol or other fermentation products. However the ability of the enzymatic conversion of a first carbon source to the intermediate, especially glucose, may be impaired by the presence of the intermediate.

For example, the typical methods used in Japanese sake brewing and  
15 alcoholic production use starch without cooking. However, these techniques require some special operations such as acidification of mash (pH 3.5), which prevents contamination of harmful microorganisms. Furthermore, these methods require a longer period of the time for the saccharification and fermentation than the present invention. In addition, these methods require complex process steps such  
20 as dialysis of a fermented broth and are too cumbersome to utilize in the general production of products via fermentation.

The use of soluble dextrans and glucose as feed-stock in fermentations have various drawbacks, including high processing cost, problems associated with viscosity and oxygen transfer. In addition, in comparison to the present invention,  
25 these methods produce lower yields of the desired products and more problems associated with the formation of by-products. Indeed, the costs of converting starch or biomass to dextrans are substantial and involve high energy input, separate reactor tanks, more time, a detailed bioprocess operation, incomplete saccharification, back-reaction, and enzymes associated with the typical pre-  
30 fermentation saccharification step. These problems have led to a number of attempts to provide methods for conversion directly to starch within one reaction vessel or container and at lower temperatures. Biotransformation of a

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carbohydrate source to 1,3-propanediol in mixed cultures is described in U.S. Patent No. 5,599,689 (Haynie, *et al.*). The method described by Haynie *et al.*, involves mixing a glycerol (*i.e.*, an intermediate) producing organism with a diol producing organism (*i.e.*, an end-product), contacting the mixed culture medium  
5 with a carbon substrate and incubating the mixed culture medium to produce the desired end-product, 1,3-propanediol. In U.S. Patent No. 4,514,496, Yoshizuma describes methods that involve maintaining the concentration of the material in the slurry relative the mashing liquid to produce alcohol by fermentation without cooking (*i.e.*, without high temperature liquefaction before saccharization. Nonetheless,  
10 these methods lack the efficiency and economical advantages provided by the present invention.

The present invention provides methods for producing end-products, including organic acids (*e.g.*, gluconic acid, ascorbic acid intermediates, succinic acid, citric acid, acetic acid, gluconic acid, and lactic acid), amino acids, antibiotics,  
15 enzymes and organic solvents (*e.g.*, 1,3-propanediol, butanediol, and acetone), glycerol, ethanol are provided. In some preferred embodiments, the methods comprise the steps of contacting at least one carbon substrate with at least one substrate converting enzyme to produce at least one intermediate; and contacting at least one intermediate with an intermediate producing enzyme in a reactor  
20 vessel, wherein at least one intermediate is substantially completely bioconverted an end-product. In some preferred embodiments, this bioconversion is achieved by microorganisms. By maintaining a low concentration of the intermediate in a conversion medium, the intermediate's catabolite repressive and/or enzymatic inhibitive effects are altered (*e.g.*, reduced). The present invention also provides  
25 various levels of intermediate concentration, substrates, intermediates and steps of converting the intermediate to the desired end-product (*e.g.*, ethanol).

### Definitions

Unless defined otherwise herein, all technical and scientific terms used  
30 herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Various references (See *e.g.*, Singleton, *et al.*, DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY, 2D ED., John Wiley and

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Sons, New York [1994]; and Hale and Marham, THE HARPER COLLINS DICTIONARY OF BIOLOGY, Harper Perennial, NY [1991]) provide general definitions of many of the terms used herein. Furthermore, all patents and publications, including all sequences disclosed within such patents and publications, referred to herein are  
5 expressly incorporated by reference.

Although any methods and materials similar or equivalent to those described herein find use in the practice of the present invention, preferred methods and materials are described herein. Numeric ranges are inclusive of the numbers defining the range. Unless otherwise indicated, nucleic acids are written left to right  
10 in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation, respectively. It is to be understood that this invention is not limited to the particular methodology, protocols, and reagents described, as these may vary.

The headings provided herein are not limitations of the various aspects or  
15 embodiments of the invention that can be had by reference to the specification as a whole. Furthermore, the terms defined immediately below are more fully defined by reference to the Specification as a whole.

As used herein, the term "carbon substrate" refers to a material containing at least one carbon atom which can be enzymatically converted into an intermediate  
20 for subsequent conversion into the desired carbon end-product. Exemplary carbon substrates include, but are not limited to biomass, starches, dextrans and sugars.

As used herein, "biomass" refers to cellulose- and/or starch-containing raw materials, including but not limited to wood chips, corn stover, rice, grasses, forages, perrie-grass, potatoes, tubers, roots, whole ground corn, cobs, grains,  
25 wheat, barley, rye, milo, brans, cereals, sugar-containing raw materials (e.g., molasses, fruit materials, sugar cane or sugar beets), wood, and plant residues. Indeed, it is not intended that the present invention be limited to any particular material used as biomass. In preferred embodiments of the present invention, the raw materials are starch-containing raw materials (e.g., cobs, whole ground corns,  
30 corns, grains, milo, and/or cereals, and mixtures thereof). In particularly preferred embodiments, the term refers to any starch-containing material originally obtained from any plant source.

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As used herein, "starch" refers to any starch-containing materials. In particular, the term refers to various plant-based materials, including but not limited to wheat, barley, potato, sweet potato, tapioca, corn, maize, cassava, milo, rye, and brans. Indeed, it is not intended that the present invention be limited to any particular type and/or source of starch. In general, the term refers to any material comprised of the complex polysaccharide carbohydrates of plants, comprised of amylose and amylopectin, with the formula  $(C_6H_{10}O_5)_x$ , wherein "x" can be any number.

As used herein, "cellulose" refers to any cellulose-containing materials. In particular, the term refers to the polymer of glucose (or "cellobiose"), with the formula  $(C_6H_{10}O_5)_x$ , wherein "x" can be any number. Cellulose is the chief constituent of plant cell walls and is among the most abundant organic substances in nature. While there is a  $\beta$ -glucoside linkage in cellulose, there is an  $\alpha$ -glucoside linkage in starch. In combination with lignin, cellulose forms "lignocellulose."

As used herein, the term "corn solids" refers to ground materials from corn, including but not limited to kernels, bran and cobs.

As used herein, "intermediate" refers to a compound that contains at least one carbon atom into which the carbon substrates are enzymatically converted. Exemplary intermediates include, but are not limited to pentoses (e.g., xylose, arabinose, lyxose, ribose, ribulose, xylulose); hexoses (e.g., glucose, allose, altrose, mannose, gulose, idose, galactose, talose, psicose, fructose, sorbose, and tagatose); and organic acids thereof.

As used herein, the term "enzymatic conversion" refers to the modification of a carbon substrate to an intermediate or the modification of an intermediate to an end-product by contacting the substrate or intermediate with an enzyme. In some embodiments, contact is made by directly exposing the substrate or intermediate to the appropriate enzyme. In other embodiments, contacting comprises exposing the substrate or intermediate to an organism that expresses and/or excretes the enzyme, and/or metabolizes the desired substrate and/or intermediate to the desired intermediate and/or end-product, respectively.

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As used herein, the term "starch hydrolyzing enzyme " refers to any enzyme that is capable of converting starch to the intermediate sugar (e.g., a hexose or pentose).

As used herein, "monosaccharide" refers to any compound having an empirical formula of  $(CH_2O)_n$ , wherein n is 3-7, and preferably 5-7. In some embodiments, the term refers to "simple sugars" that consist of a single polyhydroxy aldehyde or ketone unit. The term encompasses, but is not limited to such compounds as glucose, galactose, and fructose.

As used herein, "disaccharide" refers to any compound that comprises two covalently linked monosaccharide units. The term encompasses, but is not limited to such compounds as sucrose, lactose and maltose.

As used herein, "oligosaccharide" refers to any compound having 2 - 10 monosaccharide units joined in glycosidic linkages. In some preferred embodiments, the term refers to short chains of monosaccharide units joined together by covalent bonds.

As used herein, "polysaccharide" refers to any compound having multiple monosaccharide units joined in a linear or branched chain. In some preferred embodiments, the term refers to long chains with hundreds or thousands of monosaccharide units. Some polysaccharides, such as cellulose have linear chains, while others (e.g., glycogen) have branched chains. Among the most abundant polysaccharides are starch and cellulose, which consist of recurring glucose units (although these compounds differ in how the glucose units are linked).

As used herein, "culturing" refers to fermentative bioconversion of a carbon substrate to the desired end-product within a reactor vessel. In particularly preferred embodiments, culturing involves the growth of microorganisms under suitable conditions for the production of the desired end-product(s).

As used herein, the term "saccharification" refers to converting a directly unusable polysaccharide to a useful sugar feed-stock for bioconversion or fermentative bioconversion.

As used herein, the term "fermentation" refers to the enzymatic and anaerobic breakdown of organic substances by microorganisms to produce simpler organic products. In preferred embodiments, fermentation refers to the utilization of

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carbohydrates by microorganisms (e.g., bacteria) involving an oxidation-reduction metabolic process that takes place under anaerobic conditions and in which an organic substrate serves as the final hydrogen acceptor (*i.e.*, rather than oxygen). Although fermentation occurs under anaerobic conditions, it is not intended that the term be solely limited to strict anaerobic conditions, as fermentation also occurs in the presence of oxygen.

As used herein, the terms "substantially all consumed" and "substantially all bioconverted" refer to the maintenance of a low level of intermediate in a conversion medium which adversely affects the enzymatic inhibition, oxygen transfer, yield, byproduct minimization or catabolite repression effects of the intermediate (e.g., a hexose), upon the ability of the intermediate converting enzyme to convert the intermediate to the end-product or another intermediate and/or the ability of the substrate converting enzyme to convert the substrate to the intermediate.

As used herein, the terms "bioconversion" and "bioconverted" refer to contacting a microorganism with the carbon substrate or intermediate, under conditions such that the carbon substrate or intermediate is converted to the intermediate or desired end-product, respectively. In some embodiments, these terms are used to describe the production of another intervening intermediate in *in vitro* methods in which biocatalysts alone are used. In some preferred embodiments, the terms encompass metabolism by microorganisms and/or expression or secretion of enzyme(s) that achieve the desired conversion.

As used herein, the terms "conversion media" and "conversion medium" refer to the medium/media in which the enzymes and the carbon substrate, intermediate and end-products are in contact with one another. These terms include, but are not limited to fermentation media, organic and/or aqueous media dissolving or otherwise suspending the enzymes and the carbon substrate, intermediate and end-products. In some embodiments, the media are complex, while in other preferred embodiments, the media are defined.

As used herein, the term "end-product" refers to any carbon-source derived molecule product which is enzymatically converted from the intermediate. In particularly preferred embodiments, the methods of the present invention are used

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in order to produce a "desired end-product" (*i.e.*, the product that is intended to be produced through the use of these methods). In particularly preferred embodiments, the term refers to an alcohol, particularly ethanol.

As used herein, "low concentration" refers to a concentration level of a  
5 compound that is less than that would result in the production of detrimental effects due to the presence of the compound. In particularly preferred embodiments, the term is used in reference to the concentration of a particular intermediate below which the detrimental effects of catabolite suppression and/or enzyme inhibition are observed. In some embodiments, the term refers to the concentration level of a  
10 particular intermediate above which triggers catabolite repression and/or enzymes inhibition by substrate and/or products.

As used herein, the phrase "maintained at a level below which triggers catabolite repression effects" refers to maintaining the concentration of an intermediate to below that level which triggers catabolite repression.

15 As used herein, the term "reduces catabolite repression" means conditions under which the effects of catabolite repression are produced. In preferred embodiments, the term refers to conditions in which the intermediate concentration is less than that threshold which triggers catabolite repressive effects.

As used herein, the term "reduces enzyme inhibition" means conditions  
20 under which the inhibition of an enzyme is reduced as compared to the inhibition of the enzyme under usual, standard conditions. In preferred embodiments of the present invention, the term refers to conditions in which the concentration of an intermediate, substrate and/or product of the enzyme reaction is less than that threshold which triggers enzyme inhibition.

25 As used herein, the term "substrate converting enzyme" refers to any enzyme that converts the substrate (*e.g.*, granular starch) to an intermediate, (*e.g.*, glucose). Substrate converting enzymes include, but are not limited to alpha-amylases, glucoamylases, pullulanases, starch hydrolyzing enzymes, and various combinations thereof.

30 As used herein, the term "intermediate converting enzyme" refers to any enzyme that converts an intermediate (*e.g.*, D-glucose, D-fructose, etc.), to the desired end-product. In preferred embodiments, this conversion is accomplished

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through hydrolysis, while in other embodiments, the conversion involves the metabolism of the intermediate to the end-product by a microorganism. However, it is not intended that the present invention be limited to any particular enzyme or means of conversion. Indeed, it is intended that any appropriate enzyme will find use in the various embodiments of the present invention.

As used herein, "yield" refers to the amount of end-product or intermediate produced using the methods of the present invention. In some preferred embodiments, the yield produced using the methods of the present invention is greater than that produced using methods known in the art. In some embodiments, the yield refers to the volume of the end-product or intermediate, while in other embodiments, the term is used in reference to the concentration of the end-product or intermediate in a composition.

As used herein, "byproduct formation" refers to the production of products that are not desired. In some preferred embodiments, the present invention provides methods that avoid or reduce the production of byproducts, as compared to methods known in the art.

As used herein, the term "enzymatic inhibition" refers to loss of enzyme activity by either physical or biochemical effects on the enzyme. In some embodiments, inhibition results from the effects of the product formed by the enzyme activity, while in other embodiments, inhibition results from the action of the substrate or intermediate on the enzyme.

As used herein, "enzyme activity" refers to the action of an enzyme on its substrate. In some embodiments, the enzyme activity is quantitated using means to determine the conversion of the substrate to the intermediate, while in other embodiments, the conversion of the substrate to the end-product is determined, while in still further embodiments, the conversion of the intermediate to the end-product is determined.

As used herein, the term "enzyme unit" refers to the amount of enzyme which converts 1 micromole of substrate per minute to the substrate product at optimum assay conditions (unless otherwise noted). In some embodiments, commercially available enzymes (e.g., SPEZYME®, DISTALLASE®, OPTIMAX®; Genencor International) find use in the methods of the present invention.

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As used herein, the term "glucoamylase unit" (GAU) is defined as the amount of enzyme required to produce one micromole of glucose per minute under assay conditions of 40° C. and pH 5.0 or under the alternative assay conditions of 25° and pH 7.0.

5       As used herein, the term "glucose oxidase unit" (GOU) is defined as the amount of enzyme required to oxidize one micromole of D-glucose per minute under assay conditions of 25° C. and pH 7.0, to gluconic acid.

As used herein, the term "catalase units" (CU) is defined as the amount of enzyme required to decompose 1 micromole of hydrogen peroxide per minute  
10       under assay conditions of 25° C. and pH 7.0.

As used herein, one AG unit (AGU) is the amount of enzyme which splits one micromole of maltose per minute at 25° C. and pH 4.3. In some embodiments of the present invention, a commercially available liquid form of glucoamylase (OPTIDEX® L-400; Genencor International) with an activity of 400 GAU per ml is  
15       used. In alternative embodiments, a commercially available liquid form of glucoamylase (AMG NOVO 150) has an activity of 150 AGU per ml finds use.

As used herein, the terms "starch hydrolyzing unit" and "raw starch hydrolyzing unit" (RHU) are defined as being the amount of enzyme required to produce one gram of glucose per minute from starch, under the assay conditions of  
20       25° C. and pH 5.0.

As used herein, "carbon end-product" means any carbon product produced from the carbon intermediate, wherein the substrate contains at least one carbon atom (*i.e.*, a carbon substrate).

As used herein, "carbon intermediate" refers to the carbon-containing  
25       compounds that are produced during the conversion of a carbon-containing substrate to a carbon end-product.

As used herein, "enzymatically controlled" means regulating the amount of carbon intermediate produced from the carbon substrate by altering the amount or activity of the enzyme used in the reaction.

30       As used herein, "microorganism" refers to any organism with cells that are typically considered to be microscopic, including such organisms as bacteria, fungi (yeasts and molds), rickettsia, and protozoa. It is not intended that the present

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invention be limited to any particular microorganism(s) or species of microorganism(s), as various microorganisms and microbial enzymes are suitable for use in the present invention. It is also not intended that the present invention be limited to wild-type microorganisms, as microorganisms and microbial enzymes  
5 produced using recombinant DNA technologies also find use in the present invention.

As used herein, "microbial enzyme" refers to any enzyme that is produced by a microorganism. As used herein, a "microbial intermediate-converting enzyme" is an enzyme that converts an intermediate to an end-product, while a "microbial  
10 substrate-converting enzyme" is an enzyme that converts a substrate to an intermediate or directly converts a substrate to an end-product (*i.e.*, there is not intermediate compound).

As used herein, the term "ethanologenic microorganism" refers to a microorganism with the ability to convert a sugar or oligosaccharide to ethanol.  
15 Ethanologenic microorganisms are known in the art and include ethanologenic bacteria. The microorganisms are ethanologenic by virtue of their ability to express one or more enzymes that individually or together, convert a sugar to ethanol.

As used herein, the terms "ethanol producer" and "ethanol producing organism" refer to any organism or cell that is capable of producing ethanol from a  
20 hexose or a pentose. Generally, ethanol producing cells contain an alcohol dehydrogenase and pyruvate decarboxylase.

As used herein, "antimicrobial" refers to any compound that kills or inhibits the growth of microorganisms.

As used herein, the term "linked culture" refers to a fermentation system that  
25 employs at least two cell cultures, in which the cultures are added sequentially. In most embodiments of linked systems, a primary culture or a set of primary cultures is grown under optimal fermentation conditions for the production of a desired intermediate (*i.e.*, the intermediate is released into the culture media to produce a "conditioned medium"). Following the fermentation of the primary culture, the  
30 conditioned medium is then exposed to the secondary culture(s). The secondary cultures then convert the intermediate in the conditioned media to the desired end-product. In some embodiments of the present invention, the primary cultures are

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typically glycerol producers and the secondary cultures are 1,3-propanediol producers.

As used herein, "mixed culture" refers to the presence of any combination of microbial species in a culture. In some preferred embodiments, the mixed culture is grown in a reaction vessel under conditions such that the interaction of the individual metabolic processes of the combined organisms results in a product which neither individual organism is capable of producing. It is not intended that the present invention be limited to mixed cultures comprising a particular number of microbial species.

As used herein, "conditioned media" refers to any fermentation media suitable for the growth of microorganisms that has been supplemented by organic byproducts of microbial growth. In preferred embodiments of the present invention, conditioned media are produced during fermentation of linked cultures wherein glycerol producing cells secrete glycerol into the fermentation media for subsequent conversion to 1,3-propanediol.

As used herein, "oxygen uptake rate" ("OUR") refers to the determination of the specific consumption of oxygen within the reactor vessel. Oxygen consumption can be determined using various on-line measurements known in the art. In one embodiment, the OUR (mmol/(liter\*hour)) is determined by the following formula:

$$\left( \frac{\text{Airflow (standing liters per minute)}}{\text{Fermentation weight (weight of the fermentation broth in kilograms)}} \times \text{supply O}_2 \times \text{broth density} \times (\text{a constant to correct for airflow calibration at 21.1 C instead of standard 20.0 C}) \right) - \left( \frac{\text{airflow}}{\text{fermentation weight}} \times \left[ \frac{\text{offgas O}_2}{\text{offgas N}_2} \right] \times \text{supply N}_2 \times \text{broth density} \times \text{constant} \right)$$

As used herein, "carbon evolution rate" ("CER") refers to the determination of how much CO<sub>2</sub> is produced within the reactor vessel during fermentation. Usually, since no CO<sub>2</sub> is initially or subsequently provided to the reaction vessel, any CO<sub>2</sub> is assumed to be produced by the fermentation process occurring within the reaction vessel. "Off-gas CO<sub>2</sub>" refers to the amount of CO<sub>2</sub> measured within the reactor vessel, usually by mass spectroscopic methods known in the art.

As used herein, the term "enhanced" refers to improved production of proteins of interest. In preferred embodiments, the present invention provides

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enhanced (*i.e.*, improved) production and secretion of a protein of interest. In these embodiments, the "enhanced" production is improved as compared to the normal levels of production by the host (*e.g.*, wild-type cells). Thus, for heterologous proteins, basically any expression is enhanced, as the cells normally do not  
5 produce the protein.

As used herein, the terms "isolated" and "purified" refer to a nucleic acid or amino acid that is removed from at least one component with which it is naturally associated.

## 10 DETAILED DESCRIPTION OF THE INVENTION

The present invention provides means for the production of desired end-products of *in vitro* and/or *in vivo* bioconversion of biomass-based feed stock substrates, including but not limited to such materials as starch and cellulose. In particularly preferred embodiments, the methods of the present invention do not  
15 require gelatinization and/or liquefaction of the substrate. In particularly preferred embodiments, the present invention provides means for the production of ethanol. In some particularly preferred embodiments, the present invention provides means for the production of ethanol directly from granular starch, in which altered catabolite repression is involved.

20 In preferred embodiments, the present invention provides dramatic improvements in the process for directly converting a commonly available carbon substrate (*e.g.*, biomass and/or starch) into an intermediate, preferably, an intermediate that is readily convertible into a multitude of desired end-products, including alcohols such as ethanol. In particularly preferred embodiments, the  
25 present invention provides means for dramatically improving the processes for directly converting granular starch into glucose, an intermediate readily convertible into a ethanol.

In alternative embodiments, the present invention provides means for dramatic improvements in the process for converting starch or cellulose into  
30 glucose, which in turn is converted into the desired end-product. By maintaining the presence of the intermediate at a low concentration within the conversion media, overall efficiency of the production is improved. In some embodiments, enzymatic

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inhibition and/or catabolite repression, oxygen uptake demand, and/or byproduct formation are reduced. In additional preferred embodiments, the present invention provides means for dramatic improvements in the non-cooking conversion of granular starch into glucose, which in turn is converted into the desired end-product.

5 In some preferred embodiments, the maintenance of minimal intermediate concentrations is achieved by maintaining the concentration of the intermediate at a low concentration. In one embodiment, the concentration of the intermediate is less than or equal to 0.25% by weight volume of the medium (e.g., 0.25% to 0.00001% by weight volume). In other embodiments, the concentration of the intermediate is  
10 less than or equal to 0.20%, 0.10%, 0.05%, or 0.01% by weight volume (e.g., 0.20% to 0.00001%, 0.10% to 0.00001%, 0.05% to 0.00001%, 0.01% to 0.00001%, respectively). Alternatively, the intermediate concentration is maintained at less than or equal to a concentration of 2.0  $\mu$ molar in the conversion media. In another embodiment, the concentration is maintained at less than or equal to 1.0  $\mu$ molar. In  
15 still another embodiment, the concentration of the intermediate is maintained at a concentration of less than or equal to 0.75  $\mu$ molar. In any event, maintaining a low concentration means maintaining the concentration of the intermediate below the threshold that results in enzyme inhibition (i.e., enzyme inhibitive effects), catabolite repression (i.e., catabolite repressive effects).

20 In further embodiments, the maintenance of a minimal concentration is achieved by maintaining the rate of conversion of the substrate to the intermediate at less than or equal to the rate of conversion of the intermediate to the end-product. While it is recognized that the conversion of the substrate to the intermediate is necessarily rate limiting for the conversion of the intermediate to the  
25 end-product, by providing sufficient intermediate converting enzymes for the conversion of substantially all of the intermediate produced by the first enzymatic conversion from the carbon substrate, substantially all of the intermediate is converted to the end-product as fast as it is converted from the starting substrate to minimize the presence of the intermediate in the conversion medium. Exemplary  
30 methods of providing such excessive intermediate conversion include providing an excess of intermediate converting enzyme, increasing the enzyme activity of the intermediate converting enzyme, and/or decreasing the activity of the substrate

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converting enzyme to convert the intermediate to end-product as quickly as it is converted from the substrate. As the actual rate of conversion is contemplated to vary with the specific end product produced, some variation in the amount and experimentation in determining the amount are contemplated. However guidelines  
5 for making these determinations are provided herein.

In some embodiments of the present invention, the conversion or consumption rate of the intermediate was determined by the calculating the amount of organism present in the mixed media, taking into consideration the other physical parameters of the mixed media, and multiplying that amount by the generally known  
10 conversion rate. This provides a rate of conversion of the intermediate, (e.g., glucose), to the end-product. In some embodiments, this conversion of the intermediate to the desired end product is by conversion or bioconversion of the intermediate to the end-product by a naturally occurring organism or one mutated to provide such bioconversion. Another embodiment of the conversion from  
15 intermediate to end product involves the use of an enzymatic conversion by a known enzyme to the desired end-product using known enzymatic conversion methods. For example, in some embodiments, the conversion of glucose to a desired end product (e.g., propanediol, succinic acid, gluconic acid, lactic acid, amino acid, antimicrobials, ethanol, ascorbic acid intermediates and /or ascorbic  
20 acid) is accomplished by the addition of an amount of an enzyme known to convert glucose to the specified end product desired.

Once the conversion rate of the intermediate to the desired end product is determined, the limit of the conversion of the carbon substrate to the intermediate can be determined in the same manner. By calculating the upper limit of the  
25 intermediate to end product conversion, the conversion rate of the carbon substrate to intermediate can be determined, the main consideration being that the intermediate concentration levels in the conversion media are maintained at a sufficiently low level to adversely effect the normally catabolite repressive/enzymatic inhibitory effects of the intermediate. In one embodiment, this is accomplished by  
30 maintaining the conversion rate of the intermediate to the end product in excess or equal to the rate of conversion of the carbon substrate to the intermediate. Thus, the present invention provides means for increasing the conversion rate to the end

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product, as well as means for restricting the conversion of the carbon substrate to the intermediate.

Another method for determining whether the rate of conversion of the intermediate to the end product is greater than or equal to the production of the intermediate from the carbon substrate is to measure the weight percentage of the intermediate in the reactor vessel. The amount of the intermediate present in the reactor vessel can be determined by various known methods, including, but not limited to direct or indirect measurement of the amount of intermediate present in the reactor vessel. Direct measurement can be by periodic assays of the reactor vessel, using assays known to identify the amount of intermediate and or end-product in the vessel. In addition, direct measurement of the amounts of intermediates within the reactor vessel include on-line gas, liquid and/or high performance liquid chromatography methodologies known in the art

Indirect measurement of the levels of intermediate or end-products produced can be assessed by the measurement of oxygen uptake or carbon dioxide production, using methods known in the art (e.g., by determining the oxygen uptake rate and/or the carbon evolution rate).

### Substrates

The substrates of the present invention are carbon-based compounds that can be converted enzymatically to intermediate compounds. Suitable substrates include, but are not limited to processed materials that contain constituents which can be converted into sugars (e.g., cellulosic biomass, glycogen, starch and various forms thereof, such as corn starch, wheat starch, corn solids and wheat solids). During the development of the present invention good results were obtained with corn starch and wheat starch, although other sources, including starches from grains and tubers (e.g., sweet potato, potato, rice and cassava starch) also find use with the present invention. Various starches are commercially available. For example, corn starches are available from Cerestar, Sigma, and Katayama Chemical Industry Co. (Japan); wheat starches are available from Sigma; sweet potato starch is available from Wako Pure Chemical Industry Co. (Japan); and

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potato starch is available from Nakari Chemical Pharmaceutical Co. (Japan). A particularly useful carbon substrate is corn starch. In some embodiments of the present invention, granular starch is used in a slurry having a percentage of starch between about 20% and about 35%. Preferably, the starch is in a concentration  
5 between about 10% and about 35%. In some particularly preferred embodiments, the range for percent starch is between 30% and 32%. In addition to granular starch, other carbon substrate sources find use in the present invention include, but are not limited to biomass, polysaccharides, and other carbon based materials capable of being converted enzymatically to an intermediate.

10 Fermentable sugars can be obtained from a wide variety of sources, including lignocellulosic material. Lignocellulose material can be obtained from lignocellulosic waste products (e.g., plant residues and waste paper). Examples of suitable plant residues include but are not limited to any plant material such as stems, leaves, hulls, husks, cobs and the like, as well as corn stover, bagasses,  
15 wood, wood chips, wood pulp, and sawdust. Examples of paper waste include but are not limited to discarded paper of any type (e.g., photocopy paper, computer printer paper, notebook paper, notepad paper, typewriter paper, and the like), as well as newspapers, magazines, cardboard, and paper-based packaging materials. The conditions for converting sugars to ethanol are known in the art. Generally, the  
20 temperature is between about 25 ° C. and 35 ° C (e.g., between 25° and 35°, and more particularly at 30° C). Useful pH ranges for the conversion medium are provided between about 4.0 and 6.0, between 4.5 and 6.0, and between pH 5.5 and 5.8. However, it is not intended that the present invention be limited to any particular temperature and/or pH conditions as these conditions are dependent  
25 upon the substrate(s), enzyme(s), intermediate(s), and/or end-product(s) involved.

### Enzymes

In some preferred embodiments of the present invention, enzymes that are substrate-converting enzymes (i.e., enzymes that are able to first convert the  
30 carbon substrate into the carbon intermediate), and intermediate converting enzymes (i.e., enzymes that are able to convert the resulting intermediate into an intervening intermediate and/or the desired end-product) both find use in the

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present invention. Enzymes that find use in some embodiments of the present invention to convert a carbon substrate to an intermediate include, but are not limited to alpha-amylase, glucoamylase, starch hydrolyzing glucoamylase, and pullulanase. Enzymes that find use in the conversion of an intermediate to an end-product depend largely on the actual desired end-product. For example enzymes useful for the conversion of a sugar to 1,3-propanediol include, but are not limited to enzymes produced by *E. coli* and other microorganisms. For example enzymes useful for the conversion of a sugar to lactic acid include, but are not limited to those produced by *Lactobacillus* and *Zymomonas*. Enzymes useful for the conversion of a sugar to ethanol include, but are not limited to alcohol dehydrogenase and pyruvate decarboxylase. Enzymes useful for the conversion of a sugar to ascorbic acid intermediates include, but are not limited to glucose dehydrogenase, gluconic acid dehydrogenase, 2,5-diketo-D-gluconate reductase, and various other enzymes. Enzymes useful for the conversion of a sugar to gluconic acid include, but are not limited to glucose oxidase and catalase.

In some preferred embodiments, the alpha-amylase used in some methods of the present invention is generally an enzyme which effects random cleavage of alpha-(1-4) glucosidic linkages in starch. In most embodiments, the alpha-amylase is chosen from among the microbial enzymes having an E. C. number E. C. 3.2.1.1 and in particular E. C. 3.2.1.1-3. In some preferred embodiments, the alpha-amylase is a thermostable bacterial alpha-amylase. In most particularly preferred embodiments, the alpha-amylase is obtained or derived from *Bacillus* species. Indeed, during the development of the present invention good results were obtained using the SPEZYME® alpha-amylase obtained from *Bacillus licheniformis* (Genencor). In other embodiments, black-koji amylase described in alcoholic fermentation from starch such as corn and cassava without precooking (Ueda *et al.*, J. Ferment. Technol., 50:237-242 [1980]; and Ueda *et al.*, J. Ferment. Technol., 58:237-242 [1980]) find use in the present invention.

As understood by those in the art, the quantity of alpha-amylase used in the methods of the present invention will depend on the enzymatic activity of the alpha-amylase and the rate of conversion of the generated glucose by the end-product converter. Generally an amount between 0.001 and 2.0 ml of a solution of the

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alpha-amylase is added to 1000 gm of raw materials, although in some embodiments, it is added in an amount between 0.005 and 1.5 ml of such a solution. In some preferred embodiments, it is added in an amount between 0.1 and 1.0 ml of such a solution. In further embodiments, other quantities are utilized.

5 For example, generally an amount between 0.01 and 1.0 kg of SPEZYME® FRED (Genencor) is added to one metric ton of starch. In some embodiments, the enzyme is added in an amount between 0.4 to 0.6 kg, while in other embodiments, it is added in an amount between 0.5 and 0.6 kg of SPEZYME® FRED/metric ton of starch..

10 In preferred embodiments of the present invention, the glucoamylase is an enzyme which removes successive glucose units from the non-reducing ends of starch. The enzyme can hydrolyze both the linear and branched glucosidic linkages of starch, amylose and amylopectin. In most embodiments, the glucoamylase used in the methods of the present invention are microbial enzymes. In some preferred

15 embodiments, the glucoamylase is a thermostable fungal glucoamylase, such as the *Aspergillus* glucoamylase. Indeed, during the development of the present invention, good results were obtained using the DISTALLASE® glucoamylase derived from *Aspergillus niger* (Genencor ). Glucoamylase preparations from *Aspergillus niger* have also been used without the use of precooking (See, Ueda et

20 al, Biotechnol. Bioeng., 23:291[1981]). Three glucoamylases have been selectively separated from *Aspergillus awamori* var. *kawachi* for use in hydrolyzing starch (See, Hayashida, Agr. Biol. Chem., 39:2093-2099 [1973]). Alcoholic fermentation of sweet potato by *Endomycopsis fibuligoeu* glucoamylase without cooking has also been described (Saha et al., Biotechnol. Bioeng., 25:1181-1186 [1983]). Another

25 enzyme that finds use in the present invention is glucoamylase (EC 3.2.1.3), an enzyme that hydrolyzes the alpha.-1,4-glucoside chain progressively from the non-reducing terminal end. This enzyme also hydrolyzes the alpha-1,6-glucoside chain. Glucoamylase is secreted from fungi of the genera *Aspergillus*, *Rhizopus* and *Mucor* also find use in the methods of the present invention. These enzymes

30 further find use in glucose production and quantitative determination of glycogen and starch. Glucoamylase preparations obtained from *E. fibuligera* (IFO 0111) have been used to contact sweet potato starch for alcoholic fermentation (See,

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Saha *et al.*, Biotechnol. Bioeng., 25:1181-1186 [1983]). One of this enzyme's major applications is as a saccharifying agent in the production of ethyl alcohol from starchy materials. However, as with the other glucoamylases described herein, this enzyme also finds use in the methods of the present invention.

5 Additional glucoamylases that find use in the methods of the present invention include those obtained from the genera *Rhizopus* and *Humicola*, which are characterized as having particularly high productivity and enzymatic activity. Furthermore, in comparison with the glucoamylase derived from other organisms, the *Rhizopus*-derived glucoamylase exhibits a strong action on starch and its  
10 enzymological and chemical properties including optimum pH are particularly suitable for the saccharification of cereal starch. Because of these features, the *Rhizopus*-derived glucoamylase is considered to be best suited for alcohol production using non-cooked or low-temperature cooked starch (See, U.S. Pat. No. 4,514,496 and 4,092,434). It has been noted that upon the incubation of corn  
15 starch with *Rhizopus* glucoamylase, was used in conjunction with *Rhizopus* alpha amylase, the starch degradation by glucoamylase was accelerated. While it is not intended that the present invention be limited to any particular mechanism or theory, it is believed that *Rhizopus* glucoamylase has a stronger degradation activity than *Aspergillus niger* glucoamylase preparations which also contain  $\alpha$ -  
20 amylase (See, Yamamoto *et al.*, Denpun Kagaku, 37:129-136 [1990]). One commercial preparation that finds use in the present invention is the glucoamylase preparation derived from the Koji culture of a strain of *Rhizopus niveus* available from Shin Nippo Chemical Co., Ltd. Another commercial preparation that finds use in the present invention is the commercial starch hydrolyzing composition M1 is  
25 available from Biocon India (Bangalore, India).

As understood by those in the art, the quantity of glucoamylase used in the methods of the present invention depends on the enzymatic activity of the glucoamylase (e.g., DISTILLASE® L-400). Generally, an amount between 0.001 and 2.0 ml of a solution of the glucoamylase is added to 450 gm of a slurry adjusted  
30 to 20-35% dry solids, the slurry being the liquefied mash during the saccharification and/or in the hydrolyzed starch and sugars during the fermentation. In some embodiments, the glucoamylase is added in an amount between 0.005 and 1.5 ml

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of such a solution. In some preferred embodiments, the enzyme is added at an amount between 0.01 and 1.0 ml of such a solution.

As indicated above, pullulanases also find use in the methods of the present invention. These enzymes hydrolyze alpha.-1,6-glucosidic bonds. Thus, during the  
5 saccharification of the liquefied starch, pullulanases remove successive glucose units from the non-reducing ends of the starch. This enzyme is capable of hydrolyzing both the linear and branched glucosidic linkages of starch, amylose and amylopectin.

Additional enzymes that find use in the present invention include starch  
10 hydrolyzing (RSH) enzymes, including *Humicola* RSH glucoamylase enzyme preparation (See, U.S. Patent No. 4,618,579). This *Humicola* RSH enzyme preparation exhibits maximum activity within the pH range of 5.0 to 7.0 and particularly in the range of 5.5 to 6.0. In addition, this enzyme preparation exhibits maximum activity in the temperature range of 50° C to 60° C. Thus, in each of the  
15 steps of the present invention in which this enzyme is used, the enzymatic solubilization of starch is preferably carried out within these pH and temperature ranges.

In some embodiments, *Humicola* RSH enzyme preparations obtained from the fungal organism strain *Humicola grisea* var. *thermoidea* find use in the methods  
20 of the present invention. In some particularly preferred embodiments, these *Humicola* RSH enzymes are selected from the group consisting of ATCC (American Type Culture Collection) 16453, NRRL (USDA Northern Regional Research Laboratory) 15219, NRRL 15220, NRRL 15221, NRRL 15222, NRRL 15223, NRRL 15224, and NRRL 15225, as well as genetically altered strains derived from these  
25 enzymes.

Additional RSH glucoamylases that find use in the methods of the present invention include *Rhizopus* RSH glucoamylase enzyme preparations. In some  
embodiments, the enzyme obtained from the Koji strain of *Rhizopus niveus* available from Shin Nihon Chemical Co., Ltd., Ahjyo, Japan, under the tradename  
30 "CU CONC" is used. Another useful enzyme preparation is a commercial digestive from *Rhizopus* available from Amano Pharmaceutical under the tradename "GLUCZYME" (See, Takahashi et al., J. Biochem., 98:663-671 [1985]). Additional

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enzymes include three forms of glucoamylase (EC 3.2.1.3) of a *Rhizopus* sp., namely "Gluc1" (MW 74,000), "Gluc2" (MW 58,600) and "Gluc 3" (MW 61,400). Gluc1 was found to be 22-25 times more effective than Gluc2 or Gluc3. Thus, although Gluc2 and Gluc3 find use in the present invention, because Gluc1 tightly  
5 binds to starch and has an optimum pH of 4.5, Gluc1 finds particular use in the present invention. An additional RSH glucoamylase enzyme preparation for use in the present invention includes enzyme preparations sold under the designation "M1," available from Biocon India, Ltd., Bangalore, India. M1 is a multifaceted enzyme composition or mixture, as indicated by the high performance liquid  
10 chromatography spectra of Figure 1 and the SDS gel of Figure 2.

As noted above, in most embodiments, *Humicola* RSH glucoamylase enzyme preparations contain glucoamylase activity as well as a potentiating factor which solubilizes starch. The relative proportions of potentiating factor and glucoamylase activity in other RSH enzyme preparations may vary somewhat.  
15 However, with RSH glucoamylase enzyme preparations that find use in the present invention, there is usually ample potentiating factor produced along with the glucoamylase fraction. Accordingly, the activity of the RSH glucoamylase enzyme preparations is defined in terms of their glucoamylase activity.

Glucoamylase activity can also be measured for purposes of this invention in  
20 10 D.E. units for either RSH enzyme preparation or conventional glucoamylase. A "10 D.E. unit" is the amount of either type of enzyme which produces 1 micromole of glucose per minute under the assay conditions. To determine glucoamylase activity for purposes of this invention, one-tenth ml of enzyme preparation, diluted if necessary, containing 0.06 units to 1.1 units is added to 0.9 ml of substrate solution  
25 preheated at 50°C for 5 minutes. The substrate solution consists of 40 parts by volume 0.25M sodium acetate buffer (pH 5.5) and 50 parts by volume 4% by weight 10 D.E. maltodextrin in water. The substrate solution is kept at 50°C for 5 minutes before the enzyme solution is added. After 10 minutes, the reaction is quenched by pouring into a preheated 16 mm test tube and heating in a 100°C water bath for 6  
30 minutes. Glucose concentration is determined by any convenient method (e.g., glucose reagent kit No. 15-UV from Sigma Chemical Co. or with an instrument such as the Technicon Autoanalyzer).

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A particularly useful enzymatic composition includes a mixture of glucoamylase (e.g., DISTILLASE®) and RSH (e.g., M1). The amount of the glucoamylase useful in this combination is in the range of .2 to about 1.0 GAU units of glucoamylase per gram of granular solids. A more useful amount of  
5 glucoamylase is between about 0.75 to .5 GAU per gram of solids. The range of starch hydrolyzing enzyme (M1) present in this mixture ranges from 0.2 starch hydrolyzing units (RSHU) to about 1.0 RSHU per gram of solids. One particularly useful mixture includes about 0.6 GAU DISTILLASE® per gram of corn solids and 0.2 RSHU M1 per gram of corn solids.

10 In addition to the use of enzymatic compositions containing the above described enzymes, the present invention provides methods in which a microorganism is exposed to a substrate and uses the substrate to produce the desired end-product. Thus, in some embodiments, contacting the substrate or intermediate with a fungal, bacterial or other microorganism that produces the  
15 desired end-product is used to convert the substrate or intermediate to the desired intermediate or end-product.

In preferred embodiments of the present invention, once the carbon source is enzymatically converted to the intermediate, it is converted into the desired end-product by the appropriate methodology. Conversion is accomplished via any  
20 suitable method (e.g., enzymatic or chemical). In one preferred embodiment, conversion is accomplished by bioconversion of the intermediate by contacting the intermediate with a microorganism. In alternate preferred embodiments, the respective substrate-converting enzyme and the intermediate-converting enzyme are placed in direct contact with the substrate and/or intermediate. In some  
25 embodiments, the enzyme(s) are provided as isolated, purified or concentrated preparations.

In further embodiments, the substrate and/or intermediate are placed in direct contact with a microorganism (e.g., bacterium or fungus) that secretes or metabolizes the respective substrate or intermediate. Thus, the present invention  
30 provides means for the bioconversion of a substrate to an end-product. In some embodiments, at least one intermediate compound is produced during this conversion process.

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In some embodiments, microorganisms that are genetically modified to express enzymes not normally produced by the wild-type organism are utilized. In some particularly preferred embodiments, the organisms are modified to overexpress enzymes that are normally produced by the wild-type organism.

5           Indeed, commercially available alpha-amylases and glucoamylases find use in the methods of the present invention in economically realistic enzyme concentrations. Although commonly used fermentation conditions do not utilize optimum temperatures, the pH conditions for fermentation do correspond closely to the optimum pH for commercially available saccharification enzymes (*i.e.*, the  
10   glucoamylases). In some embodiments of the present invention, complete saccharification to glucose is favored by the gradual solubilization of granular starch. Presumably, the enzyme is always exposed to low concentrations of dextrin. In addition, the removal of glucose throughout the fermentation maintains a low glucose content in the fermentation medium. Thus, glucoamylase is exposed to  
15   low concentration of glucose. In consequence, the glucoamylase is used so effectively that economically feasible dosage levels of glucoamylase are suitable for use in the methods of the present invention (*i.e.*, glucoamylase dosage of 0.05-10.0 GAU/g of starch; and preferably 0.2-2.0 GAU/g starch).

          The dosages provided above for glucoamylase only approximate the  
20   effective concentration of the enzymatic saccharification activity in the fermentation broth, as an additional proportion of the saccharification activity is contributed by the alpha-amylase. Although it is not intended that the present invention be limited to any particular mechanism or theory, it is believed that the alpha-amylase further widens the holes bored by glucoamylase on starch granules (*See, Yamamoto et al.,*  
25   *supra*). Typically, the use of commercially available alpha-amylases results in the production of significant amounts of sugars, such as glucose and maltose.

          Addition of the alpha-amylase from *Aspergillus oryzae* (*e.g.*, FUNGAMYL) to wort has been suggested to the brewing industry. This particular enzyme saccharifies dextrans to maltotriose and maltose. Thus, although the purpose of the  
30   alpha-amylase is to liquefy the starch, its saccharification propensity also makes the alpha-amylase some part of the saccharifying enzyme content. It is believed that an alpha amylase is present in the M1 composition.

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It is also contemplated that addition of the alpha-amylase from *Aspergillus oryzae* (e.g., CLARASE® L (Genencor International Inc.) to wort will find use in the brewing industry. This particular enzyme saccharifies dextrans to maltotriose and maltose. Thus, although the purpose of the alpha-amylase is to liquefy starch, its  
5 saccharification propensity also make the alpha-amylase a portion of the saccharifying enzyme content.

Furthermore, some commercially available glucoamylases contain some alpha-amylase activity. Thus, it is possible (albeit usually not practical) to ferment particulate starch in the presence solely of glucoamylase. However, it is not  
10 intended that such embodiments be excluded from the present invention. Thus, it is also contemplated that commercially available starch hydrolyzing enzymes will find use in the present invention as part of a enzyme mixture which includes starch hydrolyzing enzymes, alpha amylases and glucoamylases. In most embodiments of the methods of the present invention, an effective amount  
15 of alpha-amylase is added to a slurry of particulate starch. Those of skill in the art understand that in addition to the uncertain amount of alpha-amylase activity contributed by glucoamylase, the effective activity of the alpha-amylase may be quite different from the unit activity values given by the supplier. The activity of alpha-amylase is pH dependent, and may be different at the pH range selected for  
20 the fermentation (*i.e.*, as compared with the test conditions employed by the suppliers for their reported unit activity values). Thus, some preliminary experiments are contemplated as being sometimes necessary in order establish the most effective dosages for alpha-amylases, including those not explicitly described herein, but find use in the methods of the present invention.

25 In some most preferred embodiments, the alpha-amylase dosage range for fungal alpha-amylases is from 0.02 GAU/g (Fungal Amylase Units) to 2.0 FAU/g of starch, although in some particularly preferably embodiments, the range is 0.05-0.6 FAU/g. One "FAU" is the amount of enzyme which breaks down 5260 mg of starch per hour under a standardized set of conditions, and corresponds to approximately  
30 25 SKB units (See, Cereal Chem., 16:712-723 [1939]). In most embodiments utilizing *Bacillus* alpha-amylases, the range is 0.01 KNU/g to 0.6 KNU/g, preferably 0.05 to 0.15 KNU/g, the NU (or Novo Unit) being the amount of enzyme which

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breaks down 5.26 mg of starch per hour under a standardized set of conditions. One KNU corresponds to 1000 NU.

It is contemplated that the uncertainty as to the real activity of both the glucoamylase and the alpha-amylase in the fermenting slurry will require some preliminary investigation into the practice of some embodiments. Optimization considerations include the fact that increasing the alpha-amylase dosage with a constant glucoamylase content, increases the fermentation rate. In addition, increasing the glucoamylase dosage with a constant alpha-amylase content increases the fermentation rate. Holding the dosage of enzyme constant and/or increasing the starch content in the slurry also increase the fermentation rate. Indeed, it is contemplated that in some embodiments, the optimum alpha-amylase dosage well exceeds dosages heretofore recommended for liquefying starch; the optimum glucoamylase may well exceed dosages recommended for saccharifying syrups. However, enzyme dosage levels should not be confused with enzyme usage. Substantial proportions of the enzymes dosed into the starch slurry are available for recovery from the fermentation broth for use anew to ferment granular starch.

A further consideration arising from employment of the enzymes at fermentation temperatures is that although the enzymes exhibit low relative activity (e.g., activity of the alpha-amylase from *B. licheniformis* at fermentation temperatures is not more than about 25% of maximum activity), the low relative activity is counterbalanced by the extended duration of the 48-120 hours of fermentation, and by the extended half-life of enzymes that have not been subjected to elevated temperatures. Indeed, it has been determined that more than 90% of the enzyme activity remains after 72 hours of fermentation. It was also noted that the use of M1 resulted in at least 50% of the starch solids being hydrolyzed after 72 hours, at least 90% hydrolyzed after 72 hours and in some cases, at least 95% hydrolyzed after 72 hours.

The alpha-amylase of *B. licheniformis* (SPEZYME® AA or SPEZYME® FRED enzymes; Genencor) is sufficiently stable to withstand brief exposures to still pot temperatures. Thus, recycle of stillage can be used as a way to recycle alpha-amylase. However, recovery of enzyme in recycled stillage requires care, in

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avoiding subjecting the fermentation broth to ethanol stripping temperatures that deactivate the enzyme(s). For example, the alcohol may be vacuum stripped from the fermentation broth and such stillage recycled to recover the enzymes suitable for use in subsequent reactions.

- 5           However, as earlier described, some RSHs (e.g., the enzyme obtained from *Rhizopus*) are available that convert starch to glucose at non-cooking temperatures (e.g., 25 to 35 °C), reducing the need for exposing the enzymatic composition to still pot temperatures. This reduces the energy costs of converting the carbon substrate to the desired end-product, thereby reducing the overall costs of manufacturing.
- 10       Thus, these enzymes find particular use in the methods of the present invention.

- In preferred embodiments of the present invention, once the carbon source is enzymatically converted to the intermediate, it is converted into the desired end-product by the appropriate methodology. Conversion is accomplished via any suitable method (e.g., enzymatic or chemical). In one preferred embodiment,
- 15       conversion is accomplished by bioconversion of the intermediate by contacting the intermediate with a microorganism. In alternate preferred embodiments, the respective substrate-converting enzyme and the intermediate-converting enzyme are placed in direct contact with the substrate and/or intermediate. In some embodiments, the enzyme(s) are provided as isolated, purified or concentrated
- 20       preparations.

- The desired end-product can be any product that may be produced by the enzymatic conversion of the substrate to the end-product. In some preferred embodiments, the substrate is first converted to at least one intermediate and then converted from the intermediate to an end-product. For example, hexoses can be
- 25       bioconverted into numerous products, such as ascorbic acid intermediates, ethanol, 1,3-propanediol, and gluconic acid. Ascorbic acid intermediates include but are not limited to 2,5-diketogluconate, 2 KLG (2-keto-L-gluconate), and 5-KDG (5-keto-D-gluconate). Gluconate can be converted from glucose by contacting glucose with glucose dehydrogenase (GDH). In addition,
- 30       gluconate itself can be converted to 2-KDG (2-keto-D-gluconate) by contacting gluconate with GDH. Furthermore, 2-KDG can be converted to 2,5-DKG by contacting 2-KDG with 2-KDGH. Gluconate can also be converted to 2-KDG by

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contacting gluconate with 2KR. Glucose can also be converted to 1,3-propanediol by contacting glucose with *E. coli*. In addition, glucose can be converted to succinic acid by contacting glucose with *E. coli*.

Additional embodiments, as described herein are also provided by the  
5 present invention. In one particularly preferred embodiment of the present invention, the end-product is ethanol. In some embodiments in which glucose is an intermediate, it is converted to ethanol by contacting glucose with an ethanologenic microorganism. In contacting the intermediate with an intermediate converting enzyme, it is contemplated that isolated and/purified enzymes are placed into  
10 contact with the intermediate. In yet another embodiment, the intermediate is contacted with bioconverting agents such as bacteria, fungi or other organism that takes in the intermediate and produces the desired end-product. In some embodiments, the organism is wild-type, while in other embodiments it is mutated.

Preferred examples of ethanologenic microorganisms include ethanologenic  
15 bacteria expressing alcohol dehydrogenase and pyruvate decarboxylase, such as can be obtained with or from *Zymomonas mobilis* (See e.g., U.S. Pat. Nos. 5,028,539, 5,000,000, 5,424,202, 5,487,989, 5,482,846, 5,554,520, 5,514,583, and copending applications having U.S. Ser. No. 08/363,868 filed on Dec. 27, 1994, U.S. Ser. No. 08/475,925 filed on Jun. 7, 1995, and U.S. Ser. No. 08/218,914 filed  
20 on Mar. 28, 1994, the teachings of all of which are hereby incorporated by reference, in their entirety).

In additional embodiments, the ethanologenic microorganism expresses xylose reductase and xylitol dehydrogenase, enzymes that convert xylose to xylulose. In further embodiments, xylose isomerase is used to convert xylose to  
25 xylulose. In additional embodiments, the ethanologenic microorganism also expresses xylulokinase, an enzyme that catalyzes the conversion of xylulose to xylulose-5-phosphate. Additional enzymes involved in the completion of the pathway include transaldolase and transketolase. These enzymes can be obtained or derived from *Escherichia coli*, *Klebsiella oxytoca* and *Erwinia* species (See e.g.,  
30 U.S. Pat. No. No. 5,514,583).

In some particularly preferred embodiments, a microorganism capable of fermenting both pentoses and hexoses to ethanol are utilized. For example in

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some embodiments, a recombinant organism which inherently possesses one set of enzymes and which is genetically engineered to contain a complementing set of enzymes is used (See e.g., U.S. Pat. Nos. 5,000,000, 5,028,539, 5,424,202, 5,482,846, 5,514,583, and WO 95/13362). In some embodiments, particularly  
5 preferred microorganisms include *Klebsiella oxytoca* P2 and *E. coli* KO11.

In some embodiments, supplements are added to the nutrient medium (i.e., the culture medium), including, but not limited to vitamins, macronutrients, and micronutrients. Vitamins include, but are not limited to choline chloride, nicotinic acid, thiamine HCl, cyanocobalamin, p-aminobenzoic acid, biotin, calcium  
10 pantothenate, folic acid, pyridoxine.HCl, and riboflavin. Macronutrients include, but are not limited to  $(\text{NH}_4)_2\text{SO}_4$ ,  $\text{K}_2\text{HPO}_4$ , NaCl, and  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ . Micronutrients include, but are not limited to  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ,  $\text{ZnCl}_2 \cdot 4\text{H}_2\text{O}$ ,  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , molybdic acid (tech),  $\text{CuCl}_3 \cdot 2\text{H}_2\text{O}$ ,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , and  $\text{H}_3\text{BO}_3$ .

#### 15 **Media and Carbon Substrates**

The conversion media in the present invention must contain suitable carbon substrates. Suitable carbon substrates include, but are not limited to biomass, monosaccharides (e.g., glucose and fructose), disaccharides (e.g., lactose and sucrose), oligosaccharides, polysaccharides (e.g., starch and cellulose), as well as  
20 mixtures thereof, and unpurified mixtures from renewable feedstocks such as cheese whey permeate, cornsteep liquor, sugar beet molasses, and barley malt. In additional embodiments, the carbon substrate comprises one-carbon substrates such as carbon dioxide, or methanol for which metabolic conversion into key biochemical intermediates has been demonstrated.

25 Although it is contemplated that all of the above mentioned carbon substrates and mixtures thereof will find use in the methods of the present invention, preferred carbon substrates include monosaccharides, disaccharides, oligosaccharides, polysaccharides, and one-carbon substrates. In more particularly preferred embodiments, the carbon substrates are selected from the group  
30 consisting of glucose, fructose, sucrose and single carbon substrates such as methanol and carbon dioxide. In a most particularly preferred embodiment, the substrate is glucose.

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As known in the art, in addition to an appropriate carbon source, fermentation media must contain suitable nitrogen source(s), minerals, salts, cofactors, buffers and other components suitable for the growth of the cultures and promotion of the enzymatic pathway necessary for the production of the desired end-product (e.g., glycerol). In some embodiments, (II) salts and/or vitamin B<sub>12</sub> or precursors thereof find use in the present invention.

### Culture Conditions

Typically, cells are grown at approximately 30 °C. in appropriate media. Preferred growth media utilized in the present invention include common commercially prepared media such as Luria Bertani (LB) broth, Sabouraud Dextrose (SD) broth or Yeast Malt Extract (YM) broth. However, other defined or synthetic growth media may also be used, as appropriate. Appropriate culture conditions are well-known to those in the art.

In some embodiments, agents known to modulate catabolite repression directly or indirectly (e.g., cyclic adenosine 2':3'-monophosphate or cyclic adenosine 2':5'-monophosphate), are incorporated into the reaction media. Similarly, the use of agents known to modulate enzymatic activities (e.g., sulphites, bisulphites and alkalis) that lead to enhancement of glycerol production also find use in conjunction with or as an alternative to genetic manipulations.

Suitable pH ranges for fermentation are between pH 5.0 to pH 9.0; while the range of pH 6.0 to pH 8.0 is particularly preferred for the initial conditions of the reaction system. Furthermore, reactions may be performed under aerobic, microaerophilic, or anaerobic conditions, as suited for the organism utilized.

### Batch and Continuous Fermentations

In some preferred embodiments, the present process uses a batch method of fermentation. A classical batch fermentation is a closed system, wherein the composition of the media is set at the beginning of the fermentation and is not subject to artificial alterations during the fermentation. Thus, at the beginning of the fermentation the medium is inoculated with the desired organism(s). In this method, fermentation is permitted to occur without the addition of any components to the

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system. Typically, a batch fermentation qualifies as a "batch" with respect to the addition of the carbon source and attempts are often made at controlling factors such as pH and oxygen concentration. The metabolite and biomass compositions of the batch system change constantly up to the time the fermentation is stopped.

- 5 Within batch cultures, cells moderate through a static lag phase to a high growth log phase and finally to a stationary phase where growth rate is diminished or halted. If untreated, cells in the stationary phase eventually die. In general, cells in log phase are responsible for the bulk of production of end product or intermediate.

- A variation on the standard batch system is the "fed-batch fermentation" system, which also finds use with the present invention. In this variation of a typical batch system, the substrate is added in increments as the fermentation progresses. Fed-batch systems are useful when catabolite repression is apt to inhibit the metabolism of the cells and where it is desirable to have limited amounts of substrate in the media. Measurement of the actual substrate concentration in fed-batch systems is difficult and is therefore estimated on the basis of the changes of measurable factors such as pH, dissolved oxygen and the partial pressure of waste gases such as CO<sub>2</sub>. Batch and fed-batch fermentations are common and well known in the art.

- It is also contemplated that the methods of the present invention are adaptable to continuous fermentation methods. Continuous fermentation is an open system where a defined fermentation media is added continuously to a bioreactor and an equal amount of conditioned media is removed simultaneously for processing. Continuous fermentation generally maintains the cultures at a constant high density where cells are primarily in log phase growth.

- 25 Continuous fermentation allows for the modulation of one factor or any number of factors that affect cell growth and/or end product concentration. For example, in one embodiment, a limiting nutrient such as the carbon source or nitrogen level is maintained at a fixed rate and all other parameters are allowed to moderate. In other systems, a number of factors affecting growth can be altered continuously while the cell concentration, measured by media turbidity, is kept constant. Continuous systems strive to maintain steady state growth conditions. Thus, cell loss due to media being drawn off must be balanced against the cell

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growth rate in the fermentation. Methods of modulating nutrients and growth factors for continuous fermentation processes as well as techniques for maximizing the rate of product formation are well known in the art of industrial microbiology.

In some embodiments, the present invention is practiced using batch  
5 processes, while in other embodiments, fed-batch or continuous processes, as well as any other suitable mode of fermentation are used. Additionally, in some embodiments, cells are immobilized on a substrate as whole-cell catalysts and are subjected to fermentation conditions for the appropriate end-product production.

#### 10 **Identification and Purification of the End-Product**

Methods for the purification of the end-product from fermentation media are known in the art. For example, propanediols can be obtained from cell media by subjecting the reaction mixture to extraction with an organic solvent, distillation and column chromatography (See e.g., U.S. Pat. No. 5,356,812). A particularly good  
15 organic solvent for this process is cyclohexane (See, U.S. Pat. No. 5,008,473).

In some embodiments, the end-product is identified directly by submitting the media to high pressure liquid chromatography (HPLC) analysis. One method of the present invention involves analysis of fermentation media on an analytical ion exchange column using a mobile phase of 0.01 N sulfuric acid in an isocratic  
20 fashion.

#### **Identification and Purification of the Enzymes**

The enzyme levels in the media can be measured by enzyme assays. For example in the manufacture of 1,3-propanediol, the levels of expression of the  
25 proteins G3PDH and G3P phosphatase are measured by enzyme assays. The G3PDH activity assay relies on the spectral properties of the cosubstrate, NADH, in the DHAP conversion to G-3-P. NADH has intrinsic UV/vis absorption and its consumption can be monitored spectrophotometrically at 340 nm. G3P phosphatase activity can be measured by any method of measuring the inorganic  
30 phosphate liberated in the reaction. The most commonly used detection method used the visible spectroscopic determination of a blue-colored phosphomolybdate ammonium complex.

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Thus, although there are various superficial resemblances between the methods known in the art and the methods of the present invention, the present invention provides more comprehensive objectives that are reflected in a great number of detail features believed to be unique to practice of this invention,  
5 including notably enzyme recycling, biomass and starch recycling.

### **Recovery**

Overall, recovery of enzymes in recycled stillage requires care, in order to avoid subjecting the conversion media to temperatures that deactivate the  
10 enzymes. In one example, for the recovery of ethanol, the alcohol is vacuum stripped from the fermentation broth and the stillage is recycled, in order to recover the enzymes. In embodiment, enzymes are recovered through the use of ultrafiltration or an electrodialysis device and recycled.

### **Process Considerations**

As indicated above, fermentation of granular starch slurry has completely different characteristics than fermentation of a syrup. Generally, a concentration of about 20% solids in solution is considered the maximum sugar content in a fermentation medium, as higher concentrations create difficulties at the onset and at  
20 the end of fermentation. However, no similar limits exist in the fermentation of a starch slurry. The concentration of starch in the slurry may vary from 10 -35 %, with no discernable consequences at the onset of fermentation. Increasing starch concentration (e.g., at constant enzyme dosages) speeds up the bioconversion rate, or conversely, allows for lowering the enzyme dosages required to achieve a  
25 given bioconversion rate. In any event fermenting until the broth has 7-10% alcohol, as is prevalent in the fermentation arts, is still possible. The excess (i.e., residual) granular starch may be recovered, along with substantial amounts of enzymes and subjected to renewed fermentation. Thus, control over starch concentration is a major process parameter for practice of this invention.

30 In one preferred embodiment, means for bioconversion and fermentation of a granular starch slurry having 10-35% starch by weight are provided. In some preferred embodiments, fermentation of a 10-35% starch slurry with *E. coli* results

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in the production of residual starch when fermentation has proceeded to the intended organic acid or 1,3-propane diol content levels. However, this reaction is dependent on the microorganism and bioprocessing conditions used and, therefore, recycling of the enzymes on the starch particles occurs when the residual starch is again fermented. However, even when a 25-35% starch slurry is fermented, in preferred embodiments, the fermentation is halted before complete disappearance of the granular starch, for fermentation anew. Thus, recycling of starch is a facile way to recover enzymes for reuse.

In an alternative embodiment, means for fermentation of a granular starch slurry of 25-25% by weight are provided. Fermenting a 25-35% starch slurry with common baker's yeast will invariably result in residual starch when fermentation has proceeded to the intended alcohol content levels (e.g., 7-10%), dependent on the microorganism used and the recycling of the enzymes on the starch particles occurs when the residual starch is again fermented. However, it is not intended that the present invention be limited to this range, as other weight percentages will find use in the present invention, depending upon the substrate and/or enzyme system utilized in the methods. For example, in some embodiments, a granular starch slurry of 10-35% by weight is preferred. A particularly useful microorganism is one that is resistant to the alcohol produced by the process.

In one preferred embodiment of the present invention, the (granular) starch and microorganisms are removed together (e.g., by centrifugation or filtration). This removed starch and microorganisms are mixed with fresh granular starch and additional aliquot(s) of enzyme(s) as needed, to produce a fermentation charge for another fermentation run.

In another embodiment, bioconversion and fermentation of a corn-stover slurry having 10-35% cellulose by weight is provided. In one embodiment, fermenting a 10-35% cellulosic slurry with *E. coli* results in residual cellulosic when fermentation has proceeded to the intended organic acid or 1,3-propane diol content levels. This reaction is dependent upon the microorganism and bioprocessing conditions used. As above, recycling of the enzymes on the cellulose occurs when the residual corn-stover is again fermented. However, even when a 25-35% cellulose slurry is fermented, in some preferred

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embodiments, the fermentation is halted before the complete disappearance of the stover, for fermentation anew. Thus, recycling stover is a facile way to recover enzymes for reuse.

In yet another preferred embodiment, the granular starch or corn stover and  
5 microorganisms are removed together (e.g., by centrifugation or filtration). This mixture of removed granular starch or corn stover and microorganisms is mixed with fresh granular starch or corn stover and additional aliquot(s) of enzyme(s) as needed, to produce a fermentation charge for another fermentation run.

As recognized by those of skill in the art, engineering trade-offs are  
10 contemplated in arriving at optimum process details; these trade-offs are contemplated to vary, depending upon each particular situation. Nonetheless, the methods provided herein provide the necessary teachings to make such trade-offs to obtain optimum processes. For example, to achieve the most rapid fermentation reasonable, high starch or cellulosic content, and high enzymes dosage are  
15 indicated. But, the consequential rapid fermentation tails off into generation of a level of nutrients in the fermentation broth, when then dictates recovery of the nutrients, or, alternatively that fermentation be halted at a relatively low end-product (e.g., alcohol) content. However, in situations where relatively low fermentation rates are acceptable, then (with high starch content slurries) enzyme dosage is  
20 relatively low and nutrient losses are held to levels heretofore accepted by the fermentation arts. In cases where maximum yield of end-product (e.g., alcohol) is a principal objective, then low starch content slurries, moderate alpha-amylase dosage, and high glucoamylase dosage find use in the present invention. However, it is not intended that the present invention be limited to any particular method  
25 design.

As indicated herein, the present invention saves considerable thermal energy. However, just as the starting substrate (e.g., starch) is never subjected to the thermal conditions used for liquefactions, the substrate is not thermally sterilized. Thus, it is contemplated that in some embodiments, the starting  
30 substrate (e.g., granular starch) adds contaminating microorganisms to the fermentation medium. Thus, in some embodiments, it is advantageous to seed the fermentation medium with a large number of the product-producing microorganisms

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that are associated with recycled substrate (e.g., starch). By greatly outnumbering the contaminants, the recycled microorganisms overwhelm any contaminating microorganisms, thereby dominating the fermentation, resulting in the production of the desired end-product. Thus, in some embodiments, the method involves

5    seeding the fermentation medium with the great number of the ethanol producing microorganism that are likely to accompany the recycled granular starch. Through their great numbers, the recycled microorganisms overwhelm any contaminating microorganisms, thereby dominating the fermentation, as is, of course, desired.

In some embodiments, the quantities of yeast initially charged into the

10   fermentation vat may be in accord with prior art practices for ethanol fermentation, and can vary widely since the yeast cells will multiply during the course of the fermentation. Recycling of yeast cells is not necessary, although may be performed. In some embodiments, the yeast is removed from the residual starch particles prior to recycling of the residual starch. However, it is noted once again

15   that practice of the present invention does not necessarily require a thermal treatment of the starch (*i.e.*, thermal conditions that would heat sterilize the starch). Thus, as with bacteria, it is advisable in some embodiments of the present invention to charge relatively large proportions of yeast cells into the fermentation in order to help overcome the likelihood of (inadvertent) contamination. In addition, in some

20   embodiments, antimicrobials are added to the fermentation medium to suppress growth of contaminating microorganisms. In further embodiments, cold sterilization techniques are utilized with the materials involved in the methods.

In most preferred embodiments, the practice of the present invention controls the fermentation rate by releasing metabolizable sugars to the microorganisms

25   (e.g., yeast) at a controlled rate and maintaining the concentration of the intermediate (e.g., glucose) at a level that does not trigger enzyme inhibition or catabolite repression. This approach is very different from what was done prior to the development of the present invention. Indeed, the prior art suggests treating solid starch with enzymes prior to fermentation and/or including enzymes in the

30   fermentation medium to conserve energy and/or to improve fermentation efficiency. However, these teachings do not alter the character of the fermentation so as to avoid the adverse effects of catabolite repression and/or enzymatic inhibition. The

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present invention also provide means to counter the adverse effects of producing undesired by-products from glucose. The present invention also provides means to conserve energy, particularly in comparison with prior art methods involving high temperature starch liquefaction. Indeed, the present invention provides means to  
5 conserve more thermal energy than other methods. The present invention provides methods that operate with high fermentation efficiency, in part because product losses due to starch retrogradation, incomplete saccharification, and incomplete fermentation of fermentables are reduced. The ability to tailor the fermentation rate through control of starch concentration and enzyme content and proportions  
10 includes the capability of creating a fermentation broth product with minimal carbohydrate content.

As indicated above, in some embodiments, the quantities of microorganisms and enzymes initially charged into the fermentation vat or bioreactor are in accord with prior art practices for the fermentation or bioconversion of various products.  
15 These quantities will vary, as the microbial cells multiply during the course of the fermentation whereas enzymes used for bioconversion will have a limited half-life. Although in some embodiments, recycling of microorganisms is utilized, in many embodiments, it is not required for the practice of the present invention. In contrast, in particularly preferred embodiments, it is desirable to recycle enzymes (although it  
20 is not intended that the present invention be limited to methods which require the recycling of enzymes).

Thus, in some embodiments, the microbes are removed from the residual starch or biomass particles prior to recycling of the residual starch or biomass. However, it is again noted that practice of the present invention does not  
25 necessarily require thermal treatment of the starting substrate (e.g., starch). Thus, in some embodiments, the starting substrate is heat-sterilized, while in other embodiments, it is not. Therefore, in some embodiments, the fermentation/bioconversion is conducted in the presence of a relatively large proportion of microorganisms, in order to overcome the effects of any  
30 contamination. In alternative embodiments, antimicrobials are added to the fermentation medium to suppress growth of contaminating microorganisms. In additional embodiments, cold sterilization techniques, UV radiation, 65°C

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pasteurization are used to sterilize the starting (e.g., substrate) materials. However, biomass poses no problem regarding sterilization of fermentation vats or bioreactors.

Use of starch as the starting material does not only address the above  
5 shortcomings of currently used methods, but has three additional significant benefits in terms of the raw material cost of corn starch vs. D-glucose, reduction of substrate and/or product based inhibition of enzymes employed in the bioconversion, and a concomitant significant reduction in the requirement of high enzyme dosages.

10 Various other examples and modifications of the description and Examples are apparent to a person skilled in the art after reading the disclosure without departing from the spirit and scope of the invention; it is intended that all such examples or modifications be included within the scope of the appended claims. All publications and patents referenced herein are hereby incorporated by reference  
15 in their entirety.

## EXPERIMENTAL

The following examples are provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention and  
20 are not to be construed as limiting the scope thereof. Indeed, it is contemplated that these teachings will find use in further optimizing the process systems described herein.

In the experimental disclosure which follows, the following abbreviations apply: wt% (weight percent); °C (degrees Centigrade); rpm (revolutions per minute);  
25 H<sub>2</sub>O (water); dH<sub>2</sub>O (deionized water); HCl (hydrochloric acid); aa (amino acid); bp (base pair); kb (kilobase pair); kD (kilodaltons); gm (grams); µg (micrograms); mg (milligrams); ng (nanograms); µl (microliters); ml (milliliters); mm (millimeters); nm (nanometers); µm (micrometer); M (molar); mM (millimolar); µM (micromolar); U (units); V (volts); MW (molecular weight); psi (pounds per square inch); sec  
30 (seconds); min(s) (minute/minutes); hr(s) (hour/hours); Q.S. and q.s. (quantity sufficient); OD (optical density); OD<sub>280</sub> (optical density at 280 nm); OD<sub>600</sub> (optical density at 600 nm); PAGE (polyacrylamide gel electrophoresis); PBS (phosphate

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buffered saline [150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2]); Cerestar (Cerestar, a Cargill, inc., company, Minneapolis, MN); SDS (sodium dodecyl sulfate); Tris (tris(hydroxymethyl)aminomethane); w/v (weight to volume); v/v (volume to volume); ATCC (American Type Culture Collection, Rockville, MD); Difco (Difco Laboratories, Detroit, MI); GIBCO BRL or Gibco BRL (Life Technologies, Inc., Gaithersburg, MD); Genencor (Genencor International, Inc., Palo Alto, CA); Shin Nihon (Shin Nihon, Japan); BioRad (BioRad Laboratories, Hercules, CA); and LeSaffre (LeSaffre Yeast Corporation, Milwaukee, WI).

In the following Examples, additional various media and buffers known to those in the art were used, including the following:

### EXAMPLE 1

#### Fermentation of Non-Cooked Corn Mash

In this Example, experiments conducted to compare starch hydrolyzing enzyme activity with a glucoamylase on uncooked starch are described.

Fermentation experiments were carried out in 250 ml flasks that were incubated in a 30°C shaker water bath. For this experiment 112 gm of 32.1% ground corn slurry containing 0.5% dry corn steep was placed in 250 ml flasks. The pH of the slurry was about 5.7, which required no further adjustment. The desired enzymes (DISTILLASE® or Sumizyme CU; Shin Nihon) were added, along with 0.37 gm of Red Star active dry yeast (LeSaffre) to start the saccharification and fermentation. During the fermentation a sample of the beer was centrifuged and .5 ml of supernatant was added to a test tube containing .05 ml of 1.1 N H<sub>2</sub>SO<sub>4</sub> containing 5% glutaraldehyde to terminate both the fermentation and enzyme action. The sample was then diluted with 5.0 ml water and then subjected to HPLC analysis on Bio Rad HPX-87H column. The results are shown in Table 1 below.

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**Table 1.****Fermentation of Uncooked Ground Corn Comparing Sumizyme CU With Distillase**

Fermentation at 30°C With enzyme dosage as GAU per gm of corn.

Flask	Enzyme	Hours	% W/V % DP>2	% W/V DP-2	% W/V DP-1	% W/V Lactic	% W/V Glycerol	% V/V Ethanol
1	0.20 GAU/g CU	24	0.27	0	0.02	0.12	0.66	8.91
		48	0.29	0	0	0.06	0.71	13.68
		72	0.30	0	0	0.02	0.59	15.07
2	0.20 GAU/g Dist	24	0.25	0	0	0.13	0.28	3.05
		48	0.20	0	0	0.37	0.26	4.59
		72	0.19	0	0	0.49	0.24	6.10
3	0.40 GAU/g Dist	24	0.20	0	0	0.09	0.34	4.59
		48	0.21	0	0	0.12	0.38	7.24
		72	0.17	0	0	0.11	0.38	9.45
4	0.75 GAU/g Dist	24	0.22	0	0	0.08	0.40	5.51
		48	0.20	0	0	0.06	0.44	9.54
		72	0.21	0	0	0.03	0.45	12.13
5	1.00 GAU/g Dist	24	0.24	0	0	0.11	0.49	6.38
		48	0.22	0	0	0.13	0.41	10.64
		72	0.18	0	0	0.07	0.61	13.29

CU = Sumizyme CU from Shin Nihon

Dist = Distillase L-400

As indicated in the Table 1, little if any detectable glucose is found in the beer, which indicated as the starch is being hydrolyzed it quickly was converted to ethanol by the yeast. Figure 1 provides a graph showing the ethanol content of the various tests.

These results could show that a starch hydrolyzing enzyme could convert the uncooked starch much more efficiently than DISTILLASE®. The rate of fermentation seems more related to the RSU activity. The .2 GAU/gm level of CU corresponds to .590 RHU/gm, while the 1.0 GAU/gm level of DISTILLASE® corresponds to only .108 RHU/gm. The RHU/GAU ratio for DISTILLASE® is .54 whereas the RHU/GAU ratio for CU is 2.98, which shows an enzyme with a high RHU/GAU ratio can better hydrolyze uncooked starch.

15

**EXAMPLE 2****Fermentation of Ground Corn Slurry**

In these experiments, the same procedure was used for this experiment as in Example 1, except that 35.9 % ground corn slurry was used (instead of corn mash), and prior to starting the fermentation the slurry was placed in a 65°C water for one

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hour as a pasteurization step. No observed gelatinization of the slurry was observed. The enzymes tested were Sumizyme CU (Example 1), a *Rhizopus* glucoamylase preparation (M1) from Biocon assayed at 178 GAU/gm and 277 RHU/gm, and DISTILLASE® L-400 (Dist.) at 361 GAU/gm and 196 RHU/gm. Table 2 provides the conditions used for this study, and also summarizes the results.

Table 2.

## Fermentation Uncooked Starch With Separate And Enzyme Combinations

Sample	Enzyme	Level	Enzyme	Level	Hours	% W/V DP>2	% W/V DP-2	% W/V DP-1	% W/V Lactic	% W/V Glycerol	% V/V Ethanol
1	M1	.20 GAU/g			24	0.40	0.04	0.02	0.17	0.67	8.66
1					48	0.35	0.02	0.00	0.10	0.74	12.28
1					72	0.36	0.01	0.00	0.04	0.76	14.02
2	M1	.50 GAU/g			24	0.42	0.03	0.01	0.13	0.80	11.91
2					48	0.41	0.04	0.00	0.04	0.82	15.24
2					72	0.54	0.03	0.00	0.02	0.84	15.23
3	M1	.75 GAU/g			24	0.42	0.03	0.01	0.12	0.86	12.43
3					48	0.53	0.02	0.01	0.06	0.91	15.30
3					72	0.55	0.03	0.01	0.03	0.94	15.43
4	CU	.20 GAU/g			24	0.34	0.03	0.10	0.14	0.92	10.59
4					48	0.35	0.07	0.05	0.09	1.03	14.96
4					72	0.40	0.04	0.04	0.04	1.04	15.63
5	CU	.50 GAU/g			24	0.37	0.13	0.80	0.13	0.96	12.20
5					48	0.45	0.24	1.15	0.08	1.05	14.96
5					72	0.45	0.25	1.46	0.07	1.08	14.96
6	CU	.75 GAU/g			24	0.43	0.16	1.15	0.13	0.97	12.69
6					48	0.51	0.30	2.19	0.08	1.05	14.90
6					72	0.51	0.33	2.67	0.07	1.08	14.83
7	M1	.20 GAU/g	Dist	.2 GAU/g	24	0.41	0.04	0.02	0.14	0.71	9.19
7					48	0.35	0.01	0.00	0.07	0.75	13.06
7					72	0.40	0.02	0.00	0.02	0.78	15.20
8	M1	.20 GAU/g	Dist	.6 GAU/g	24	0.33	0.04	0.03	0.15	0.77	9.56
8					48	0.39	0.02	0.00	0.09	0.84	13.56
8					72	0.38	0.03	0.00	0.04	0.86	15.02
9	M1	.20 GAU/g	Dist	2.0 GAU/g	24	0.30	0.03	0.03	0.13	0.82	10.46
9					48	0.33	0.02	0.01	0.08	0.89	14.66
9					72	0.38	0.03	0.01	0.03	0.90	15.74

10

The ethanol results from the fermentations with M1 and CU are plotted in Figure 2A and 2B. At the .2 GAU/gm level for M1 the rate and yield of ethanol is less than the .5 and .75 levels indicating the .2 level is enzyme limiting. The .5 and .75 levels of M1 seem to give very similar results indicating that enzyme is no longer limiting. The results from CU similarly shows that the .2 enzyme level is somewhat limiting the fermentation, but is faster than .2 GAU/gm for M1 results. This indicates that the RHU activity is a better parameter that indicates the hydrolysis of uncooked starch. CU has about twice the RHU activity per GAU as does M1, and CU is seen to hydrolyze the uncooked starch faster at similar GAU

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levels. At the .5 and .75 GAU/gm dosage excess glucose is observed particularly at the higher enzyme level. Actually it appears that starch hydrolyzing rate is faster than the fermentation rate. These results also show that at around 15% ethanol, the ethanol seems to become toxic to the yeast since the fermentations appeared to stop.

The graph provided at Figure 2, Panel C shows the ethanol results from the fermentations where M1 was added to DISTILLASE®. As the results show, adding DISTILLASE® to a low level of M1, .2 GAU/gm, both the rate and yield of ethanol increased improving the performance of M1. These results show that by adding a glucoamylase preparation with a GSH ratio greater than 1.5 to DISTILLASE®, which has only a GSH ratio less than .6, can hydrolyze uncooked starch so that ethanol can be made by a process that eliminates the cooking step.

### EXAMPLE 3

#### Comparison of Cooked and Uncooked Corn Mash

In these experiments, fermentations were conducted similar to that described in Example 1, except 83 gm of 28.9% ground corn slurry were placed in 250 ml bottles containing a magnetic bar. The bottles were placed on a submersion magnetic stirrer in a 30°C water bath so that the mash was gently mixed during the fermentation. Combinations of DISTILLASE® and M1 were tested as shown in Table 3. These fermentation were started with .27 gm of dry yeast. After the fermentation, the beer was dried in a 65°C forced air oven to obtain what was considered the DDGS (Distillers Dry Grains plus the Solubles). In this manner a quantitative estimate of the DDGS was obtained, and the starch contend of the DDGS was obtained by a starch analysis technique. The HPLC profiles of the fermentations are also shown in Table 3.

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Table 3.

Trial	M1 GAU/g	Dist. GAU/g	Hours	HPLC Profile					
				% W/V DP>2	% W/V DP-2	% W/V DP-1	% W/V Lactic	% W/V Glycerol	% V/V Ethanol
1	0.10	0.00	24	0.23	0	0	0.08	0.28	3.40
1			48	0.26	0	0	0.46	0.56	5.66
1			72	0.27	0	0	0.62	0.33	6.95
2	0.10	0.20	24	0.24	0	0	0.11	0.41	5.70
2			48	0.27	0	0	0.09	0.46	9.21
2			72	0.32	0	0	0.04	0.48	11.44
3	0.10	0.40	24	0.19	0	0	0.14	0.52	6.87
3			48	0.26	0	0	0.12	0.61	10.87
3			72	0.27	0	0	0.05	0.63	12.98
4	0.10	0.60	24	0.22	0	0	0.15	0.59	7.79
4			48	0.26	0	0	0.12	0.67	11.93
4			72	0.29	0	0	0.05	0.69	13.63
5	0.10	1.00	24	0.18	0	0.01	0.15	0.69	9.07
5			48	0.23	0	0	0.10	0.76	12.74
5			72	0.29	0	0	0.05	0.79	14.14
6	0.20	0.00	24	0.22	0	0	0.12	0.42	5.64
6			48	0.27	0	0	0.08	0.43	8.86
6			72	0.32	0	0	0.03	0.46	11.33
7	0.20	0.20	24	0.23	0	0	0.15	0.54	7.29
7			48	0.25	0	0	0.13	0.53	11.15
7			72	0.22	0	0	0.07	0.66	13.09
8	0.20	0.40	24	0.21	0	0	0.15	0.62	8.46
8			48	0.25	0	0	0.13	0.70	12.38
8			72	0.27	0	0	0.06	0.53	13.65
9	0.20	0.60	24	0.25	0	0	0.14	0.63	9.43
9			48	0.21	0	0	0.08	0.72	13.15
9			72	0.29	0	0	0.03	0.73	14.40
10	0.20	1.00	24	0.24	0	0.02	0.14	0.75	10.32
10			48	0.25	0	0	0.08	0.78	14.12
10			72	0.31	0	0.01	0.04	0.80	14.31
11	0.40	0.00	24	0.26	0	0	0.16	0.56	8.00
11			48	0.31	0	0	0.10	0.64	12.04
11			72	0.27	0	0	0.04	0.67	13.77
12	0.40	0.20	24	0.22	0	0	0.14	0.62	9.24
12			48	0.29	0	0	0.09	0.69	13.55
12			72	0.28	0	0	0.03	0.70	14.01
13	0.40	0.40	24	0.25	0	0	0.15	0.69	10.15
13			48	0.29	0	0	0.09	0.75	13.64
13			72	0.33	0	0	0.05	0.77	14.40
14	0.40	0.60	24	0.24	0	0.02	0.14	0.73	10.89
14			48	0.31	0	0	0.09	0.79	13.84
14			72	0.34	0	0	0.04	0.78	14.19
15	0.40	1.00	24	0.26	0	0.02	0.13	0.76	11.35
15			48	0.32	0	0	0.08	0.82	14.30
15			72	0.29	0	0	0.05	0.83	14.54

At each level of M1 tested the addition of DISTILLASE® improved the fermentation rate and yield of ethanol, as shown in Figure 3, Panels A, B and C.

The starch analyses of the DDGS are shown in Table 4. From these analyses and the amount of DDGS, an estimate was then made of the amount of starch that remained unconverted in the fermenter. As indicated by these results, the addition of DISTILLASE® to M1 helps improve the hydrolysis of uncooked starch.

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Table 4.

Trial	M1 GAU/g	Dist. GAU/g	% V/V Ethanol	DDGS		% Unused Starch
				gm DS	% Starch	
1	0.10	0.00	6.95	13.93	52.37	46.75
2	0.10	0.20	11.44	8.88	26.84	15.29
3	0.10	0.40	12.98	7.29	15.60	7.29
4	0.10	0.60	13.63	6.74	9.53	4.12
5	0.10	1.00	14.14	6.68	5.60	2.40
6	0.20	0.00	11.33	9.51	33.64	20.51
7	0.20	0.20	13.09	7.53	16.84	8.13
8	0.20	0.40	13.65	6.72	8.88	3.83
9	0.20	0.60	14.40	6.37	3.75	1.53
10	0.20	1.00	14.31	6.36	2.50	1.02
11	0.40	0.00	13.77	7.00	9.43	4.23
12	0.40	0.20	14.01	6.78	8.57	3.73
13	0.40	0.40	14.40	6.38	3.12	1.28
14	0.40	0.60	14.19	6.44	2.02	0.83
15	0.40	1.00	14.54	6.41	1.61	0.66

Thus, the results obtained in these Examples indicate that adding a glucoamylase preparation with a GSH ratio greater than 1.5 to a glucoamylase with GSH ratio less than .6 can hydrolyze uncooked starch such that ethanol fermentations can be carried out on mashes that are not cooked. These results demonstrated the percent composition of a high GSH ratio glucoamylase to a low GSH ratio as low as 9% is very effective in hydrolyzing uncooked starch.

#### EXAMPLE 4

##### Influence of Stillage on the Fermentation of Cooked and Non-cooked Mash

This Example describes experiments designed to evaluate the fermentation of liquefied corn mash containing various levels of stillage compared to the fermentation of non-cooked corn mash containing various levels of stillage. The enzymes used in the fermentations were different. FERMENZYME® was used for fermenting the liquefied mash, which is a preparation that is similar to what is used commercially. For the non-cooked mash fermentation, a combination of DISTILLASE® and the RSH enzyme M1 were used.

The experiment was set up so that the corn solids would be constant while the solids from the stillage would vary. This meant that the total solids in the

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fermenters increased as the stillage increased. Thin stillage was obtained from a local dry mill ethanol plant. The thin stillage was concentrated in a vacuum rotary evaporator to 44% solids. It was necessary to concentrate the thin stillage, so that the total solids in the fermenters would be manageable. The mash composition for each fermenter is shown in Table 5.

Table 5: Fermenter Mash Composition

31% Liquefied Corn at pH 5.0, .25 gm yeast, 150 gm Total .4 GAU/gm Fermentzyme				
No	Liquefact gm	Stillage Syrup gm	Water gm	Mash % DS
1	130	0	20.0	31.1
2	130	2	18.0	31.7
3	130	5	15.0	32.5
4	130	10	10.0	34.0
5	130	15	5.0	35.5
6	130	20	0.0	36.9
31% Noncooked Corn <30 mesh at pH 5.0, .25 gm Yeast, 150 gm Total .6 GAU/gm Distillase + .19 RHU/gm M1 + GC106 equivalent				
No	Corn gm	Stillage Syrup gm	Water gm	Mash % DS
7	52.4	0	97.1	30.9
8	52.4	2	95.1	31.5
9	52.4	5	92.1	32.4
10	52.4	10	87.1	33.9
11	52.4	15	82.1	35.3
12	52.4	20	77.1	36.8
13	52.4	25	72.1	38.3
14	52.4	30	67.1	39.7

The enzyme used for the liquefied corn mash was 0.4 GAU/gm of corn liquefact of FERMENTZYME®. FERMENTZYME®, a special blend of DISTILLASE® and a fungal protease for fermenting corn mash, is commercially available from Genencor®. For the non-cooked fermentations, a combination of DISTILLASE® and M1 were used along with the equivalent amount of protease that was in the FERMENTZYME® used in the liquefied corn mash test runs. As indicated in Table 5, the solids in the fermenters varied (mash % DS). The results of the fermentations are given in Table 6, below.

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Table 6: HPLC Profile During Fermentation										
Ferm	Mash	Stillage gm	Enzyme	Hours	% W/V DP>2	% W/V DP-2	% W/V DP-1	% W/V Lactic	% W/V Glycerol	% V/V Ethanol
1	Liq	0	Fermentzyme	24	4.93	3.63	1.66	0.69	0.60	8.74
1				48	1.50	0.56	2.17	0.88	0.83	14.08
1				72	0.71	0.56	0.17	0.84	0.85	16.28
2	Liq	2	Fermentzyme	24	5.34	3.94	1.72	0.82	0.79	9.33
2				48	1.77	0.61	4.06	0.99	1.03	12.85
2				72	0.90	0.61	3.14	0.99	1.04	14.04
3	Liq	5	Fermentzyme	24	5.75	4.40	1.80	0.98	1.07	9.35
3				48	2.07	0.65	4.66	1.13	1.27	11.92
3				72	1.14	0.68	3.26	1.16	1.35	13.54
4	Liq	10	Fermentzyme	24	5.74	4.31	1.55	1.15	1.28	9.41
4				48	2.28	0.72	5.21	1.36	1.56	11.80
4				72	1.35	0.75	4.73	1.36	1.59	12.88
5	Liq	15	Fermentzyme	24	6.26	4.65	1.64	1.47	1.56	9.66
5				48	2.56	0.87	5.50	1.59	1.82	11.56
5				72	1.57	0.84	4.71	1.58	1.83	12.58
6	Liq	20	Fermentzyme	24	6.16	1.67	1.67	1.64	1.81	9.30
6				48	2.75	0.91	5.79	1.77	2.05	11.57
6				72	1.83	1.00	6.48	1.82	2.00	11.63
7	g. com	0	Distillase + M1 + GC106	24	0.34	0	0.01	0.14	0.67	11.86
7				48	0.36	0	0	0.17	0.78	15.65
7				72	0.39	0	0	0.18	0.86	17.48
8	g. com	2	Distillase + M1 + GC107	24	0.35	0	0.02	0.23	0.77	11.96
8				48	0.40	0	0	0.25	0.88	16.09
8				72	0.38	0	0	0.19	0.95	17.56
9	g. com	5	Distillase + M1 + GC108	24	0.40	0	0.02	0.38	0.88	12.07
9				48	0.42	0	0	0.38	0.99	15.99
9				72	0.43	0	0	0.31	1.07	18.03
10	g. com	10	Distillase + M1 + GC109	24	0.54	0	0.03	0.65	1.02	11.31
10				48	0.53	0	0	0.66	1.18	15.95
10				72	0.52	0	0	0.65	1.24	17.69
11	g. com	15	Distillase + M1 + GC110	24	0.70	0	0	0.83	1.18	10.22
11				48	0.66	0	0.10	0.87	1.40	15.53
11				72	0.62	0	0	0.83	1.42	17.14
12	g. com	20	Distillase + M1 + GC111	24	0.82	0	0	1.15	1.47	10.04
12				48	0.78	0	0	1.09	1.52	14.32
12				72	0.78	0	0	1.04	1.70	17.24
13	g. com	25	Distillase + M1 + GC112	24	1.04	0	0	1.48	1.78	7.71
13				48	1.01	0	0	1.40	1.77	11.40
13				72	0.99	0	0	1.42	1.97	14.72
14	g. com	30	Distillase + M1 + GC113	24	1.17	0	0	1.74	2.02	7.68
14				48	1.18	0	0	1.70	2.06	11.61
14				72	1.12	0	0	1.65	2.18	14.58

5 In commercial practice, a certain amount of the stillage is recycled for the yeast nutrient content and to help the water balance in the plant. Thus, fermentation systems that are less influenced by stillage is very desirable in industrial fermentation plants. The results of these experiments show that stillage effects the fermentation of liquefied mash more than in non-cooked mash.

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Figure 4 shows the response of ethanol to the amount of stillage added in both types of mashes. In both cases, increasing the stillage solids reduced the ethanol level. But as Figure 4 shows, the non-cooked mash is much less sensitive to stillage than the cooked mash.

5        Figure 5 shows the glucose profile after 72 hour of fermentation and the results are very striking. As the stillage solids increased in cooked mash more glucose was left unfermented, while in the non-cooked mash, essentially non-detectable levels of glucose were observed at all levels of stillage. This observation is very significant because as the starch was hydrolyzed it was immediately to  
10 ethanol by the yeast. This level of glucose build-up is very unusual. This observation is also important particularly with respect to the yeast, in that even though the glucose level is extremely low, the yeast remain very active in fermenting. In contrast, in the cooked mash, even when glucose was in ample supply, the yeast could not ferment the glucose.

15        Figure 6 is a plot of the disaccharides after 72 hours of fermentation with respect to stillage added. The disaccharide levels for the non-cooked mash were found to be essentially non-detectable throughout the range of stillage added, but in the cooked mash as the stillage level increased the disaccharides increased.

20        As indicated in Figure 7, the higher sugars (*i.e.*, oligosaccharides greater than disaccharides) provided a somewhat a similar picture, as the level of higher sugars for the cooked mash were higher with respect to stillage added than for the non-cooked mash.

Figure 8 shows the lactic acid level after 72 hours of fermentation. As indicated, the levels are higher for the cooked mash than the non-cooked mash.  
25        One consideration with lactic acid is that it is a measure of contamination. Although it is not intended that the present invention be limited to any particular theory or mechanism, it is possible that since both the glucose and disaccharide levels are always very low in the non-cooked mash, contaminating microorganisms have very little substrate to utilize.

30        Figure 9 provides a summary of the glycerol levels after 72 hour of fermentation. As indicated, again the levels are lower at the respective stillage addition levels with the non-cooked mash than with the cooked mash. A

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contributing factor for glycerol formation during fermentation with yeast is the stress the yeast is under. Generally, the more stress the yeast is under, the more glycerol that will be formed. The results in Figure 9 would indicate that at similar stillage levels, the yeast in the non-cooked mash are under less stress. But even at higher  
5 stillage levels that could be run with cooked mash, higher levels of glycerol were formed. Even with the higher glycerol level in the non-cooked mash, the yeast produced more ethanol. Thus, it appears that yeast seem to ferment more efficiently in the non-cooked mash than in the cooked mash.

10 Various other examples and modifications of the foregoing description and examples will be apparent to a person skilled in the art after reading the disclosure without departing from the spirit and scope of the invention, and it is intended that all such examples or modifications be included within the scope of the appended claims. All publications and patents referenced herein are hereby incorporated by  
15 reference in their entirety.

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## CLAIMS

1. A method for producing an alcohol as an end-product comprising the steps of:
  - a) contacting a carbon substrate and at least one substrate-converting enzyme to produce an intermediate; and
  - b) contacting said intermediate with at least one intermediate-converting enzyme, wherein said intermediate is substantially all converted by said intermediate enzyme to said alcohol.
2. The method of Claim 1, wherein said intermediate-converting enzyme is a microbial enzyme.
3. The method of Claim 2, wherein said intermediate-converting microbial enzyme is secreted by a microorganism in contact with said intermediate.
4. The method of Claim 1, wherein said substrate-converting enzyme is a microbial enzyme.
5. The method of Claim 4, wherein said substrate-converting microbial enzyme is secreted by a microorganism in contact with said substrate.
6. The method of Claim 1, wherein said intermediate-converting enzyme and said substrate-converting enzyme are produced by microorganisms of the same species.
7. The method of Claim 1, wherein said intermediate-converting enzyme and said substrate-converting enzyme are produced by microorganisms of the different species.

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8. The method of Claim 1, wherein concentration level of said intermediate is maintained at a level below that which triggers catabolite repression effects upon the conversion of said intermediate to said end-product.

9. The method of Claim 1, wherein concentration level of said intermediate is maintained at a level below that which triggers enzymatic inhibition effects upon the conversion of said intermediate to said end-product.

10. The method of Claim 1, wherein said intermediate is converted to said end-product at a rate sufficient to maintain the concentration of said at less than 0.25%.

11. The method of Claim 1, wherein said substrate is selected from the group consisting of biomass and starch.

12. The method of Claim 11, wherein said biomass comprises corn solids.

13. The method of Claim 1, wherein said intermediate is selected from the group consisting of hexoses and pentoses.

14. The method of Claim 13, wherein said hexose is glucose.

15. The method of Claim 1, wherein said contacting said substrate and substrate-converting enzyme further comprises bioconverting said substrate to produce said intermediate.

16. The method of Claim 1, wherein said alcohol end-product is ethanol.

17. The method of Claim 1, wherein the step of contacting said substrate and said at least one substrate-converting enzyme further comprises providing an amount of said substrate-converting enzyme at a concentration that produces said

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intermediate at a concentration that is less than or equal to the amount of said intermediate converted by said at least one intermediate-converting enzyme.

18. The method of Claim 1, wherein said at least one substrate-converting enzyme converts at least 50% of said substrate to said intermediate within 72 hours.

19. The method of Claim 18, wherein said at least one substrate-converting enzyme converts at least 90% of said substrate to said intermediate within 72 hours.

20. The method of Claim 19, wherein said at least one substrate-converting enzyme converts at least 95% of said substrate to said intermediate within 72 hours.

21. The method of Claim 1, wherein said at least one substrate-converting enzyme and said at least one intermediate-converting enzyme are obtained from a microorganism selected from the group consisting of *Rhizopus* and *Aspergillus*.

22. A method for producing alcohol as an end-product comprising the steps of:

- a) contacting a carbon substrate and at least one substrate-converting enzyme to produce an intermediate; and
- b) contacting said intermediate with at least one intermediate-converting enzyme, wherein said intermediate is substantially all converted by said intermediate enzyme to said alcohol end-product, and wherein the presence of said end-product does not inhibit the further production of said alcohol end-product.

23. The method of Claim 22, wherein said intermediate-converting enzyme is a microbial enzyme.

24. The method of Claim 22, wherein said intermediate-converting microbial enzyme is secreted by a microorganism in contact with said intermediate.

25. The method of Claim 22, wherein said substrate-converting enzyme is a microbial enzyme.

26. The method of Claim 22, wherein said substrate-converting microbial enzyme is secreted by a microorganism in contact with said substrate.

27. The method of Claim 22, wherein said intermediate-converting enzyme and said substrate-converting enzyme are produced by microorganisms of the same species.

28. The method of Claim 22, wherein said intermediate-converting enzyme and said substrate-converting enzyme are produced by microorganisms of the different species.

29. The method of Claim 22, wherein said alcohol end-product is ethanol.

30. A method for producing an alcohol end-product comprising the steps of:

- a) contacting a carbon substrate and at least one substrate-converting enzyme to produce an intermediate; and
- b) contacting said intermediate with at least one intermediate-converting enzyme, wherein said intermediate is substantially all converted by said intermediate enzyme to said alcohol end-product, and wherein the presence of said substrate does not inhibit the further production of said alcohol end-product.

31. The method of Claim 30, wherein said intermediate-converting enzyme is a microbial enzyme.

32. The method of Claim 30, wherein said intermediate-converting microbial enzyme is secreted by a microorganism in contact with said intermediate.

33. The method of Claim 30, wherein said substrate-converting enzyme is a microbial enzyme.

34. The method of Claim 30, wherein said substrate-converting microbial enzyme is produced is secreted by a microorganism in contact with said substrate.

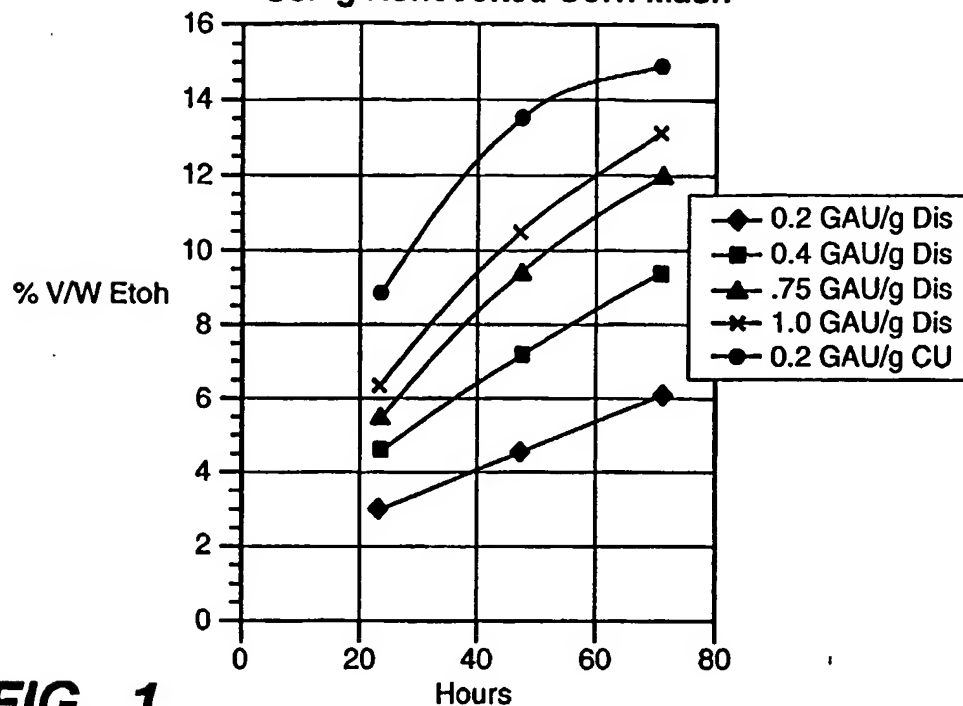
35. The method of Claim 30, wherein said intermediate-converting enzyme and said substrate-converting enzyme are produced by microorganisms of the same species.

36. The method of Claim 30, wherein said intermediate-converting enzyme and said substrate-converting enzyme are produced by microorganisms of the different species.

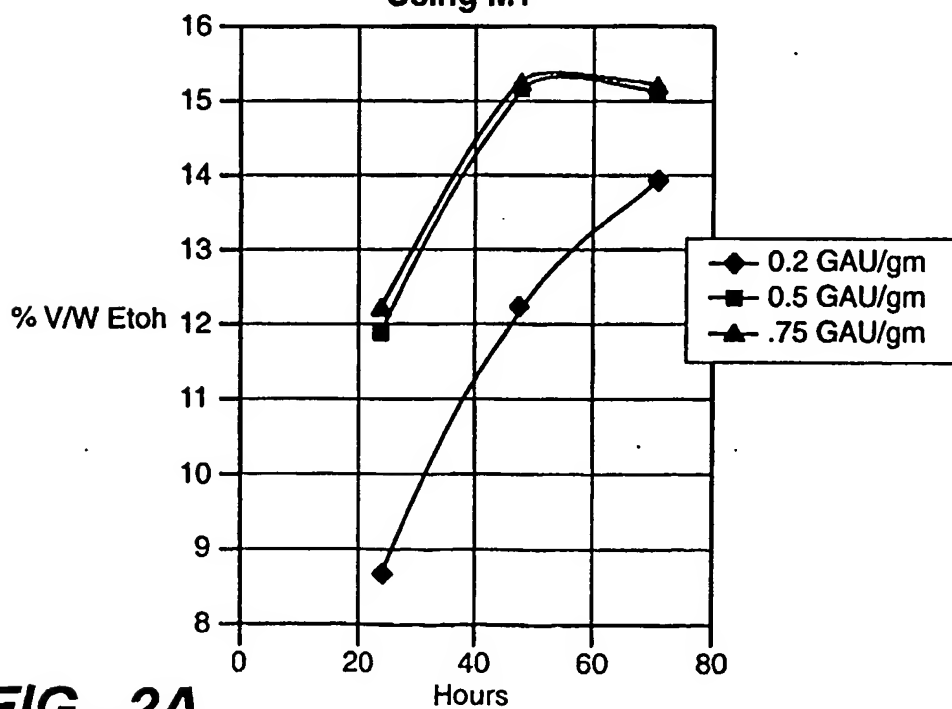
37. The method of Claim 36, wherein said alcohol end-product is ethanol.

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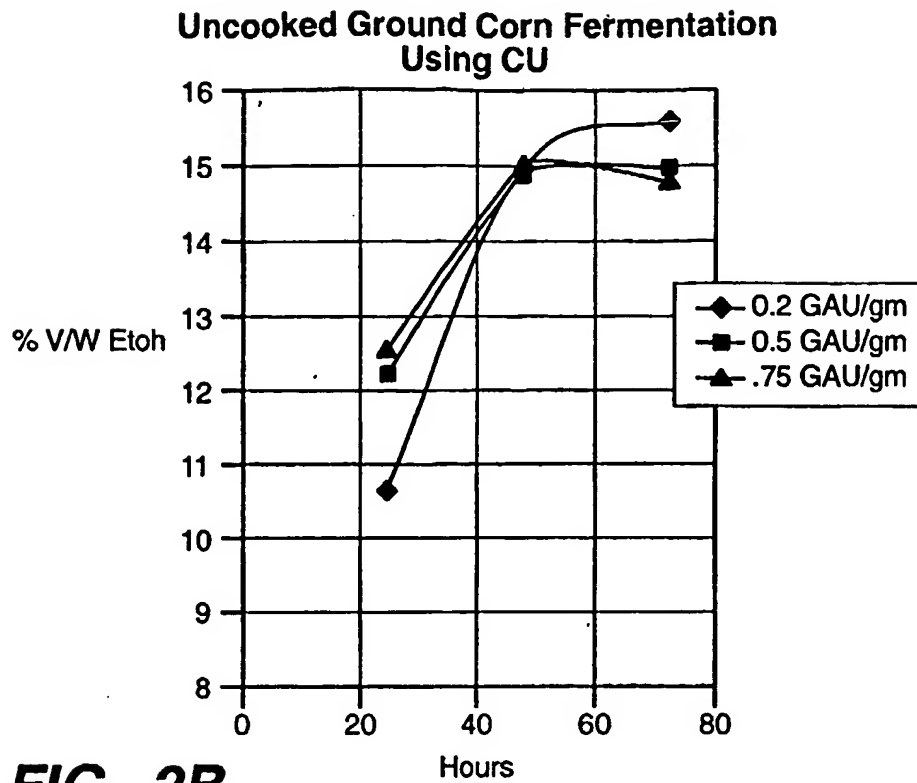
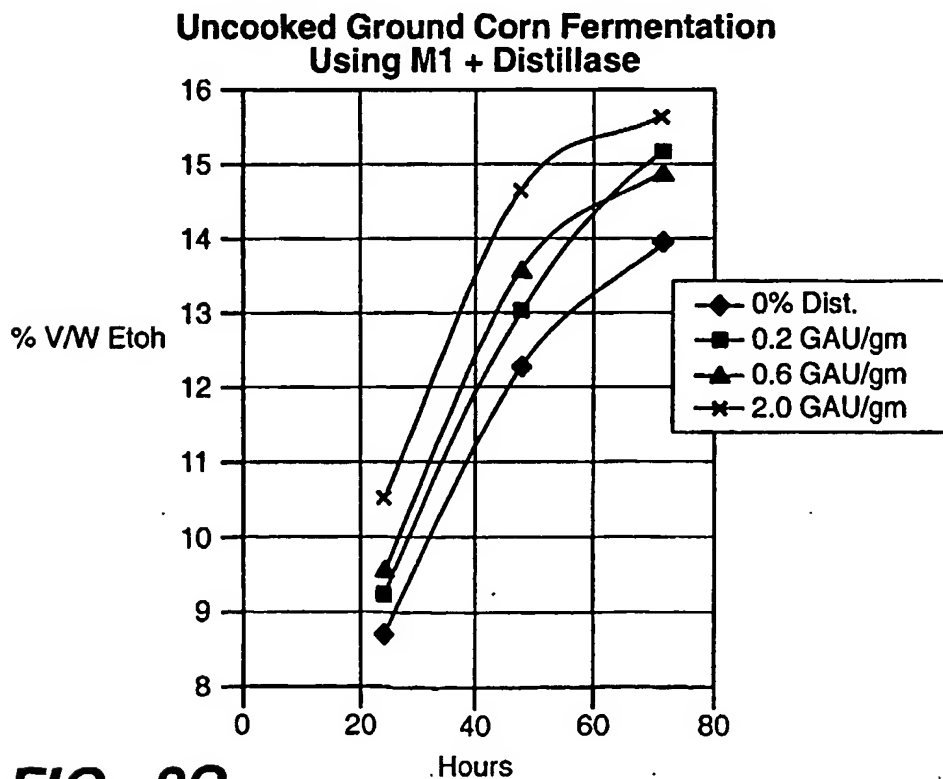
### Influence of Glucoamylase in Ethanol Fermentation Using Noncooked Corn Mash

**FIG.\_1**

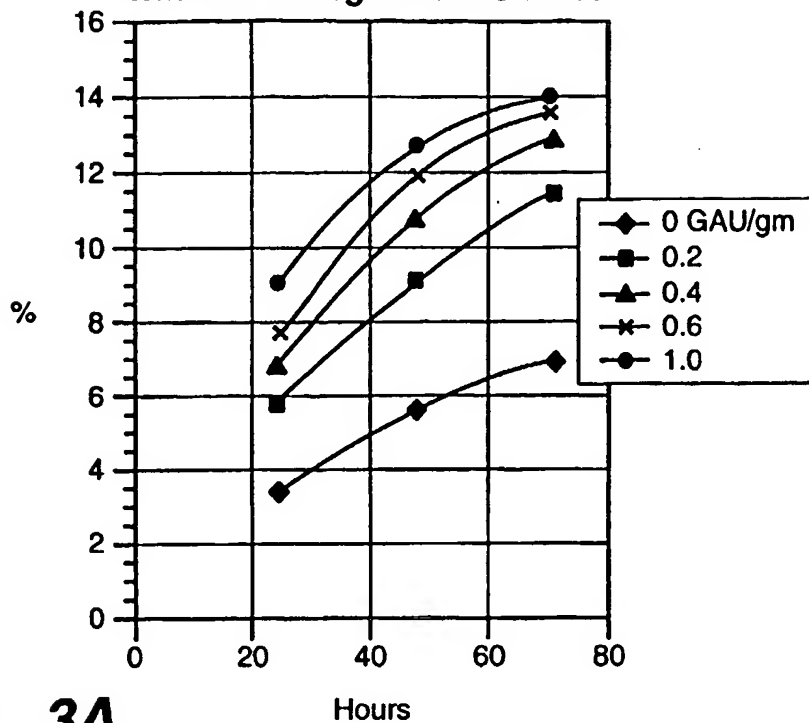
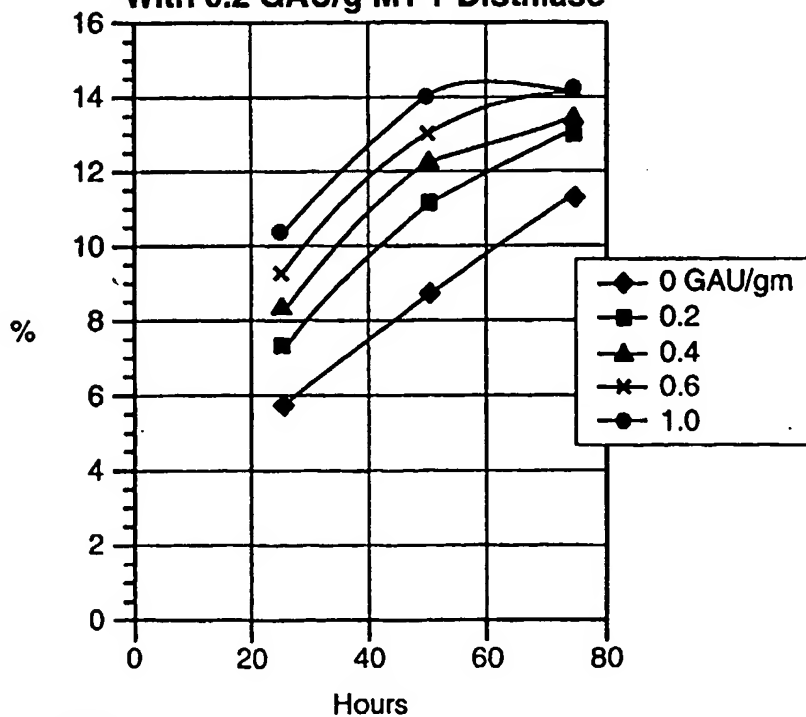
### Uncooked Ground Corn Fermentation Using M1

**FIG.\_2A**

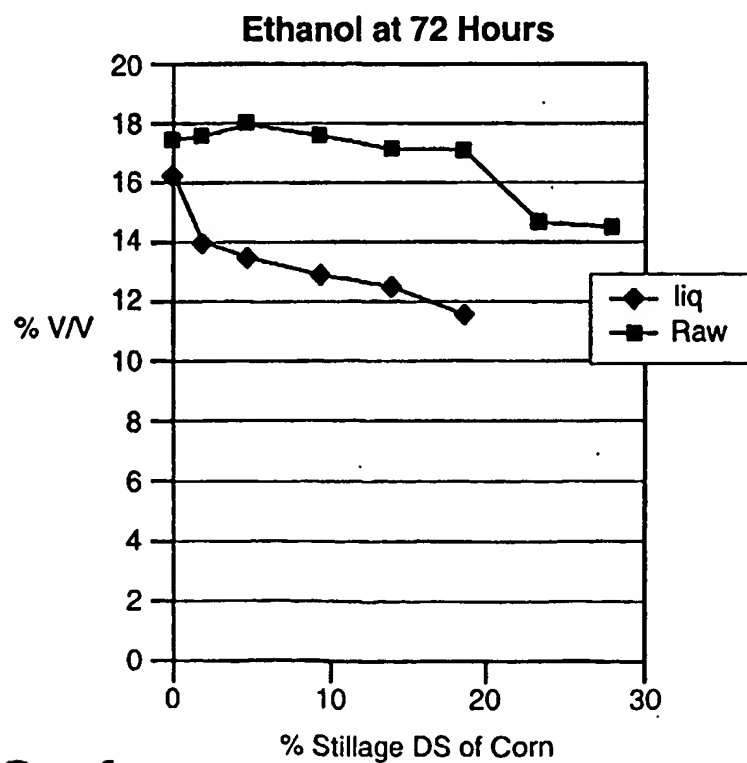
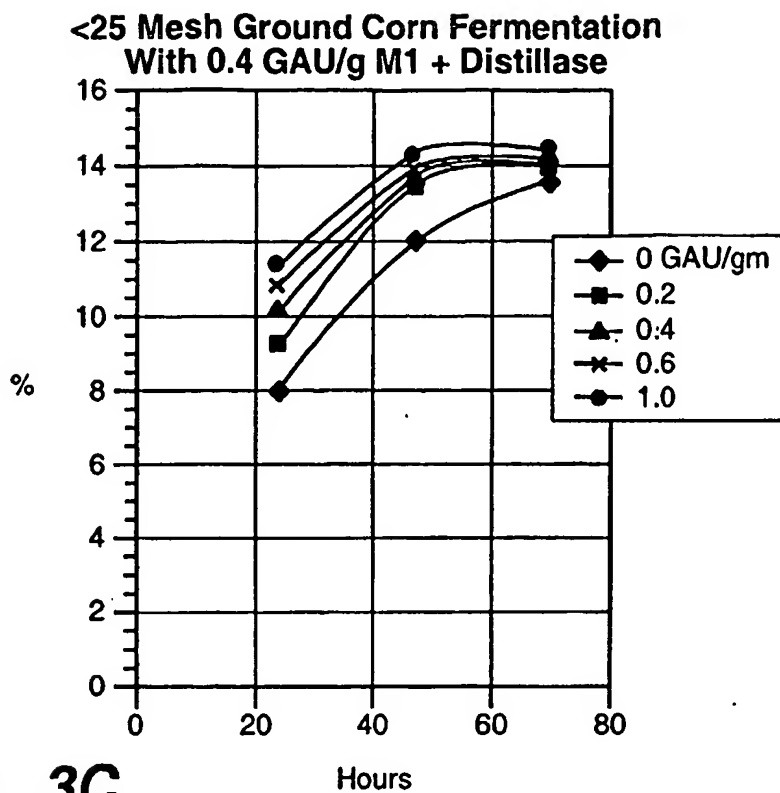
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**FIG. 2B****FIG. 2C**

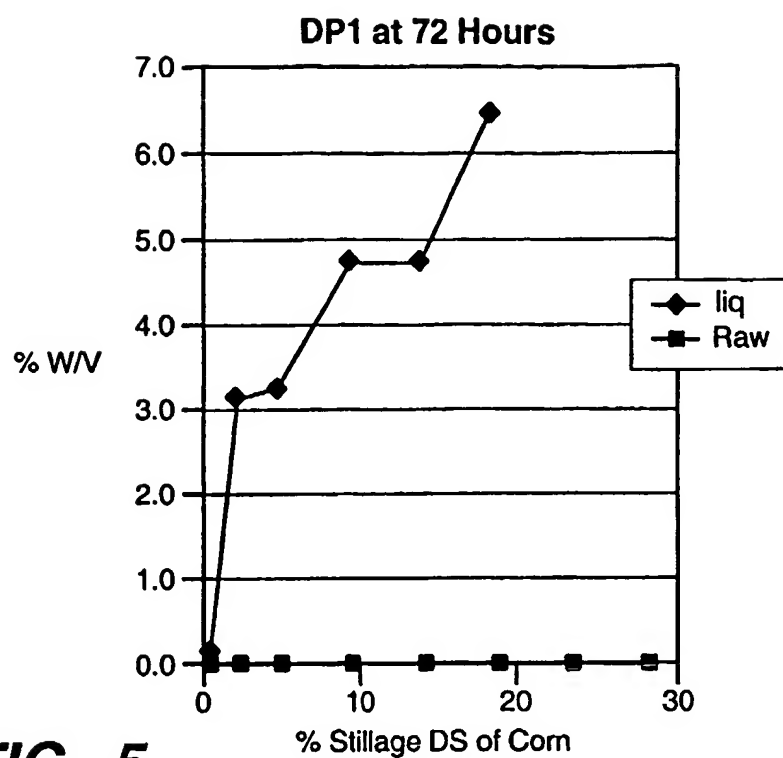
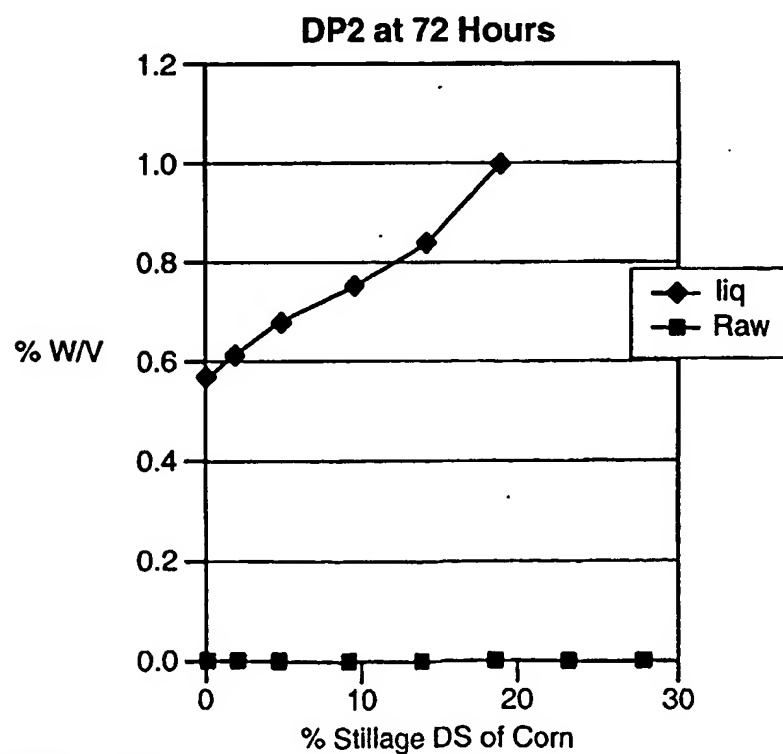
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**<25 Mesh Ground Corn Fermentation  
With 0.1 GAU/g M1 + Distillase****FIG.\_3A****<25 Mesh Ground Corn Fermentation  
With 0.2 GAU/g M1 + Distillase****FIG.\_3B**

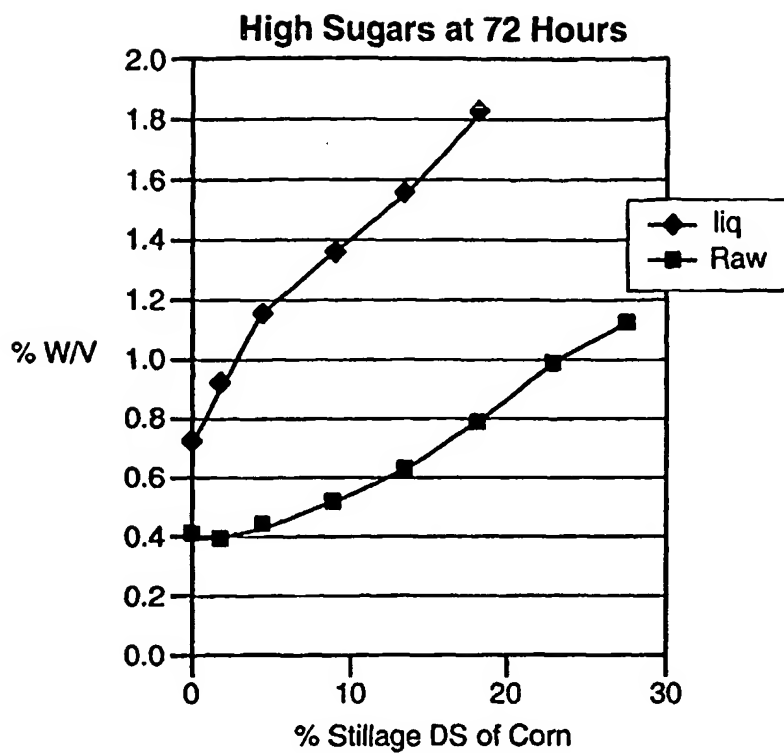
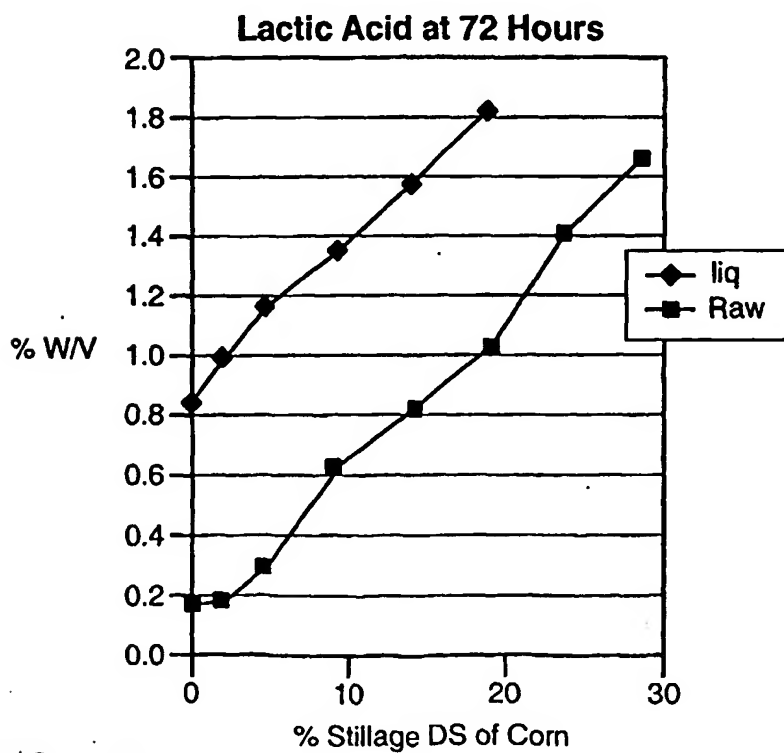
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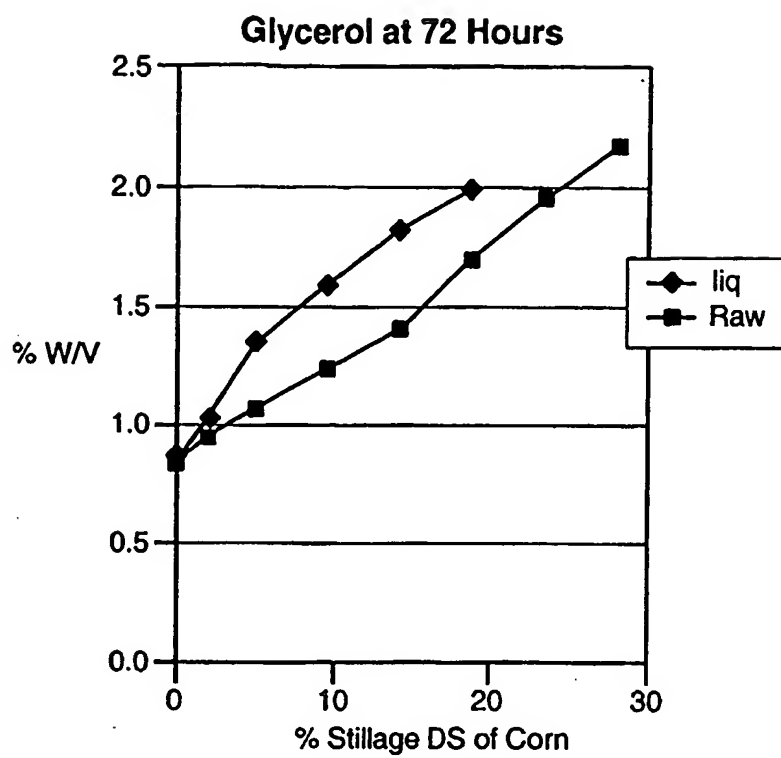
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**FIG.\_5****FIG.\_6**

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**FIG.\_7****FIG.\_8**

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**FIG.\_9**

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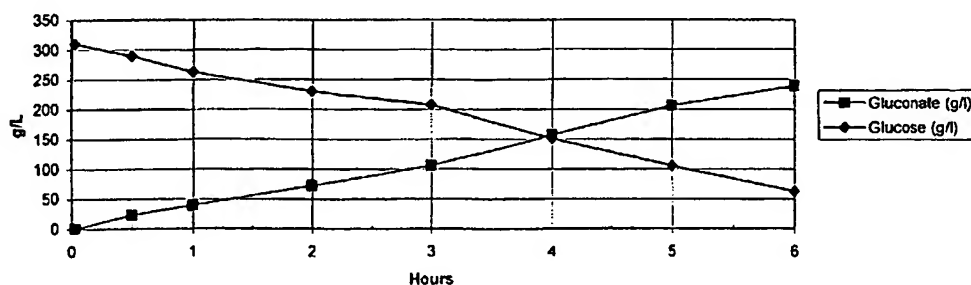
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(54) Title: METHODS FOR PRODUCING END-PRODUCTS FROM CARBON SUBSTRATES



(57) Abstract: The present invention provides means for the production of desired end-products of in vitro and/or in vivo bioconversion of biomass-based feed stock substrates, including but not limited to such materials as starch and cellulose. In particularly preferred embodiments, the methods of the present invention do not require gelatinization and/or liquefaction of the substrate.

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## **METHODS FOR PRODUCING END-PRODUCTS FROM CARBON SUBSTRATES**

### **5 FIELD OF THE INVENTION**

The present invention provides means for the production of desired end-products of *in vitro* and/or *in vivo* bioconversion of biomass-based feed stock substrates, including but not limited to such materials as starch and cellulose. In particularly preferred embodiments, the methods of the present invention do not require gelatinization and/or liquefaction of the  
10 substrate.

### **BACKGROUND OF THE INVENTION**

Industrial fermentations predominantly use glucose as feed-stock for the production of proteins, enzymes and chemicals. These fermentations are usually batch, fed-batch, or  
15 continuous, and operate under substrate-limited and minimal by-products forming conditions. These are critical operating conditions that must be controlled during fermentation in order to optimize fermentation time, yield and efficiency. Currently used methods and feed-stocks have drawbacks that reduce the efficiency of the fermentation processes.

20 Glucose is a natural, carbon based compound that is useful in a multitude of chemical and biological synthetic applications as a starting substrate. However, syrups that contain glucose purity levels of greater than 90% are relatively expensive. In addition, the presence of high glucose concentrations increases the susceptibility of the fermentation system to microbial contamination, thereby resulting in an adverse effect upon the  
25 production efficiency. Another disadvantage is that even the presence of low to moderate levels of glucose in the fermentation vat adversely affects the conversion of the glucose to the desired end product, for example by enzymatic inhibition and/or catabolite repression, and/or the growth of microorganisms. As a result, various attempts have been made to reduce the costs of industrial fermentation, particularly in utilization of less expensive  
30 substrates than glucose. However, despite the development of numerous approaches, there remains a need in the art for economical, efficiently-utilized substrates for fermentation. Indeed, there is a great need in the art for methods that utilize a less expensive starting material than glucose to more efficiently produce a desired end-product.

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**SUMMARY OF THE INVENTION**

The present invention provides means for the production of desired end-products of *in vitro* and/or *in vivo* bioconversion of biomass-based feed stock substrates, including but not limited to such materials as starch and cellulose. In particularly preferred embodiments, the methods of the present invention do not require gelatinization and/or liquefaction of the substrate.

In some preferred embodiments, the present invention provides methods for producing an end-product characterized by maintaining the intermediate concentration of the conversion at a low concentration, preferably below the threshold triggering catabolite repression and/or enzyme inhibition, so as to increase efficiency of the process by avoiding catabolic repressive and/or enzymatic inhibitive effects of the intermediate upon the enzymatic conversion of the substrate to the end-product.

In some particularly preferred embodiments, the present invention provides methods for producing an end-product, including organic acids, including but not limited to gluconic acid, ascorbic acid intermediates, succinic acid, citric acid, acetic acid, lactic acid, amino acids, and antimicrobials, as well as enzymes and organic solvents, including but not limited to 1,3-propanediol, butanol, acetone, glycerol, and ethanol. In some embodiments, the methods comprise the steps of contacting a carbon substrate and at least one substrate converting enzyme to produce an intermediate; and then contacting the intermediate with at least one intermediate producing enzyme, wherein the intermediate is substantially completely bioconverted by an end-product producing microorganism. In additional embodiments, the substrate-converting and/or intermediate-converting enzyme(s) are provided as a cell-free extract.

In some preferred embodiments, production of end-products is efficiently accomplished by maintaining a low concentration of the intermediate in a conversion medium, such that catabolite repression and/or enzyme inhibition effects associated with intermediate product formation are reduced. The present invention provides methods in various levels of intermediate concentration, substrates, intermediates and steps of converting the intermediate to ethanol are provided.

The present invention provides methods for producing end-products comprising the steps of: contacting a carbon substrate and at least one substrate-converting enzyme to produce an intermediate; and contacting the intermediate with at least one intermediate-converting enzyme, wherein the intermediate is substantially all converted by the intermediate enzyme to an end-product. In some preferred embodiments, the intermediate-converting enzyme is a microbial enzyme. In some alternative embodiments, the microbial

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enzyme is produced in by a microorganism in contact with the intermediate. In some additional embodiments, the substrate-converting enzyme is a microbial enzyme. In further embodiments, the microbial enzyme is produced by a microorganism in contact with the substrate. In still further embodiments, both the substrate-converting enzyme and the intermediate-converting enzyme are produced by a microorganism in contact with the intermediate and/or the substrate. In some embodiments, both enzymes are provided by the same species of microorganism, while in other embodiments, the enzymes are produced by microorganisms of different species. In some particularly preferred embodiments, the concentration level of the intermediate is maintained at a level below that which triggers catabolite repression effects upon the conversion of the intermediate to the end-product. In further preferred embodiments, the concentration level of the intermediate is maintained at a level below that which triggers enzymatic inhibition effects upon the conversion of the intermediate to the end-product. In still other embodiments, the intermediate is converted to the end-product at a rate sufficient to maintain the concentration of at less than 0.25% of the mixture. In some particularly preferred embodiments, the substrate is selected from the group consisting of biomass and starch. In still further embodiments, the intermediate is selected from the group consisting of hexoses and pentoses. In some preferred embodiments, the hexose is glucose. In various preferred embodiments, the end-product is selected from the group consisting of 1,3-propanediol, gluconic acid, glycerol, succinic acid, lactic acid, 2,5-diketo-D-gluconic acid, gluconate, glucose, alcohol, and ascorbic acid intermediates. In other embodiments, more than one end-product is produced. In still further embodiments, the step of contacting the substrate and at least one substrate-converting enzyme further comprises bioconverting the substrate to produce the intermediate. In some embodiments, more than one intermediate is produced. In this case, in some embodiments, the intermediate-converting enzyme(s) work on all of the intermediates, while in other embodiments, the intermediate-converting enzyme(s) work on a subset of the intermediates, while in further embodiments, the intermediate-converting enzyme(s) work on only one of the intermediates to produce at least one end-product. . In additional embodiments, the substrate-converting and/or intermediate-converting enzyme(s) are provided as a cell-free extract.

The present invention also provides methods for producing an end-product comprising the steps of contacting a carbon substrate and at least one substrate-converting enzyme to produce an intermediate; and contacting the intermediate with at least one intermediate-converting enzyme, wherein the intermediate is substantially all converted by the intermediate enzyme to an end-product, and wherein the presence of the end-product

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does not inhibit the further production of the end-product. In some embodiments, more than one intermediate is produced. In this case, in some embodiments, the intermediate-converting enzyme(s) work on all of the intermediates, while in other embodiments, the intermediate-converting enzyme(s) work on a subset of the intermediates, while in further  
5   embodiments, the intermediate-converting enzyme(s) work on only one of the intermediates to produce at least one end-product. In some embodiments, the intermediate-converting enzyme is a microbial enzyme, while in other embodiments the substrate-converting enzyme is a microbial enzyme. In some preferred embodiments, the substrate-converting and/or  
10   intermediate converting enzymes are produced by a microorganism in contact with the intermediate and/or the substrate. In some embodiments, both enzymes are provided by the same species of microorganism, while in other embodiments, the enzymes are produced by microorganisms of different species. In additional embodiments, the substrate-converting and/or intermediate-converting enzyme(s) are provided as a cell-free extract.

The present invention also provides methods for producing an end-product  
15   comprising the steps of: contacting a carbon substrate and at least one substrate-converting enzyme to produce an intermediate; and contacting the intermediate with at least one intermediate-converting enzyme, wherein the intermediate is substantially all converted by the intermediate enzyme to an end-product, and wherein the presence of the substrate does not inhibit the further production of the end-product. In some embodiments, the  
20   intermediate-converting enzyme is a microbial enzyme, while in other embodiments the substrate-converting enzyme is a microbial enzyme. In some preferred embodiments, the substrate-converting and/or intermediate converting enzymes are produced by a microorganism in contact with the intermediate and/or the substrate. In some embodiments, both enzymes are provided by the same species of microorganism, while in other  
25   embodiments, the enzymes are produced by microorganisms of different species. In some embodiments, more than one intermediate is produced. In this case, in some embodiments, the intermediate-converting enzyme(s) work on all of the intermediates, while in other embodiments, the intermediate-converting enzyme(s) work on a subset of the intermediates, while in further embodiments, the intermediate-converting enzyme(s) work on only one of  
30   the intermediates to produce at least one end-product. . In additional embodiments, the substrate-converting and/or intermediate-converting enzyme(s) are provided as a cell-free extract.

In some preferred embodiments, the contacting steps take place in a reaction vessel, including but not limited to vats, bottles, flasks, bags, bioreactors, and any other receptacle  
35   suitable for conducting the methods of the present invention.

## DESCRIPTION OF THE FIGURES

Figure 1 provides a graph showing the bioconversion of glucose to gluconic acid by the enzymes OXYGO® and FERMCOLASE® in a batch bioreactor.

5        Figure 2 provides a graph showing the bioconversion of raw corn starch to D-glucose by CU CONC RSH glucoamylase (Shin Nihon Chemicals, Japan) in a batch bioreactor.

Figure 3 provides a graph showing the bioconversion of raw corn starch to D-gluconate in the presence of CU CONC, OXYGO®, and FERMCOLASE® enzymes in a batch bioreactor.

10       Figure 4 provides a graph showing the bioconversion of starch to gluconic acid in the presence of CU CONC, OXYGO®, FERMCOLASE®, and DISTILLASE® enzymes under modified conditions in a batch bioreactor.

Figure 5 provides a graph showing the bioconversion of maltodextrin to glucose by OPTIMAX® 4060 in a batch bioreactor.

15       Figure 6 provides a graph showing results from an enzyme dosage analysis to determine the appropriate enzyme concentration for the most efficient bioconversion of glucose to gluconate.

Figure 7 provides a graph showing the bioconversion of maltodextrin to gluconate under modified enzyme dosages.

20       Figure 8 provides a graph showing the optimization of enzyme dosage to improve overall conversion of maltodextrin to gluconate.

Figure 9 provides a graph showing the bioconversion of starch to 2,5-diketo gluconic acid (DKG).

25       Figure 10, provides graph showing the bioconversion of granular starch to glucose and lactate.

Figure 11 provides a graph showing the biocatalytic conversion of granular starch to glucose and its conversion to succinate.

Figure 12 provides a graph showing the bioconversion of granular starch to glucose, its conversion to glycerol, and then to 1,3-propanediol.

30       Figure 13 provides a graph showing the bioconversion of granular starch to glucose formation, its conversion to glycerol, and then to 1,3-propanediol.

Figure 14 provides a graph showing bioconversion of granular starch to glycerol.

Figure 15 provides a graph showing bioconversion of corn starch to glucose and its conversion to 2,5-diketo-D-gluconic acid.

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Figure 16(A), provides a graph showing the biconversion of cellulose (AVICEL®) to glucose by SPEZYME® enzyme.

Figure 16(B) provides a graph showing the biocatalytic conversion of cellulose (AVICEL®) to gluconic acid by SPEZYME® ("SPE"), OXYGO® and FERMCOLASE® enzymes.

Figure 16(C) provides a graph showing the biocatalytic conversion of corn stover to gluconic acid by SPEZYME® ("SPE"), OXYGO® and FERMCOLASE® enzymes.

Figure 16(D) provides a graph showing the biocatalytic conversion of cellulose (AVICEL®) to gluconic acid by SPEZYME® ("SPE"), OXYGO® and FERMCOLASE® enzymes.

Figure 17 provides a graph showing the bioconversion of cellulose to glycerol and 1,3-propanediol.

Figure 18 provides a graph showing the bioconversion of cellulose to lactate.

Figure 19 provides a graph showing the bioconversion of cellulose to succinate.

#### BRIEF DESCRIPTION OF THE INVENTION

The present invention provides means for the production of desired end-products of *in vitro* and/or *in vivo* bioconversion of biomass-based feed stock substrates, including but not limited to such materials as starch and cellulose. In particularly preferred embodiments, the methods of the present invention do not require gelatinization and/or liquefaction of the substrate.

The present invention provides methods in which starches or biomass and hydrolyzing enzymes are used to convert starch or cellulose to glucose. In addition, the present invention provides methods in which these substrates are provided at such a rate that the conversion of starch to glucose matches the glucose feed rate required for the respective fermentative product formation. Thus, the present invention provides key glucose-limited fermentative conditions, as well as avoiding many of the metabolic regulations and inhibitions.

In some preferred embodiments, the present invention provides means for making desired end-products, in which a continuous supply of glucose is provided under controlled rate conditions, providing such benefits as reduced raw material cost, lower viscosity, improved oxygen transfer for metabolic efficiency, improved bioconversion efficiency, higher yields, altered levels of catabolite repression and enzymatic inhibition, and lowered overall manufacturing costs.

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As indicated above, there is a great need in the art for methods in which less expensive starting materials than glucose are used to efficiently produce a desired end-product. As described in greater detail herein, the present invention provides methods involving such substrates, including starch (*e.g.*, corn and wheat starch) and biomass.

5 Starch is a plant-based fermentation carbon source. Corn starch and wheat starch are carbon sources that are much cheaper than glucose carbon feedstock for fermentation. Conversion of liquefied starch to glucose is known in the art and is generally carried out using enzymes such as alpha-amylase, pullulanase, and glucoamylase. A large number of processes have been described for converting liquefied starch to the monosaccharide,  
10 glucose. Glucose has value in itself, and also as a precursor for other saccharides such as fructose. In addition, glucose may also be fermented to ethanol or other fermentation products. However the ability of the enzymatic conversion of a first carbon source to the intermediate, especially glucose, may be impaired by the presence of the intermediate.

For example, the typical methods used in Japanese sake brewing and alcoholic  
15 production use starch without cooking. However, these techniques require some special operations such as acidification of mash (pH 3.5), which prevents contamination of harmful microorganisms. Furthermore, these methods require a longer period of the time for the saccharification and fermentation than the present invention. In addition, these methods require complex process steps such as dialysis of a fermented broth and are too  
20 cumbersome to utilize in the general production of products via fermentation.

The use of soluble dextrins and glucose as feed-stock in fermentations have various drawbacks, including high processing cost, and problems associated with viscosity and oxygen transfer. In addition, in comparison to the present invention, these methods produce lower yields of the desired products and more problems associated with the formation of by-  
25 products. Indeed, the costs of converting starch or biomass to dextrins are substantial and involve high energy input, separate reactor tanks, more time, a detailed bioprocess operation, incomplete saccharification, back-reaction, and enzymes associated with the typical pre-fermentation saccharification step. These problems have led to a number of attempts to provide methods for conversion directly to starch within one reaction vessel or  
30 container and at lower temperatures. Biotransformation of a carbohydrate source to 1,3-propanediol in mixed cultures is described in US Pat. No. 5,599,689 (Haynie, *et al.*). The method described by Haynie *et al.*, involves mixing a glycerol (*i.e.*, an intermediate) producing organism with a diol producing organism (*i.e.*, an end-product), contacting the mixed culture medium with a carbon substrate and incubating the mixed culture medium to  
35 produce the desired end-product, 1,3-propanediol. In U.S. Patent No. 4,514,496,

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Yoshizuma describes methods that involve maintaining the concentration of the raw material in the slurry relative the mashing liquid to produce alcohol by fermentation without cooking (*i.e.*, without high temperature liquefaction before saccharization. Nonetheless, these methods lack the efficiency and economical advantages provided by the present invention.

5       The present invention provides methods for producing end-products, including organic acids (*e.g.*, gluconic acid, ascorbic acid intermediates, succinic acid, citric acid, acetic acid, gluconic acid, and lactic acid), amino acids, antibiotics, enzymes and organic solvents (*e.g.*, 1,3-propanediol, butanol, and acetone), glycerol, and ethanol are provided. In some preferred embodiments, the methods comprise the steps of contacting at least one  
10   carbon substrate with at least one substrate converting enzyme to produce at least one intermediate; and contacting the at least one intermediate with an intermediate producing enzyme (typically within a reaction vessel of any suitable type), wherein the at least one intermediate is substantially completely bioconverted an end-product. In some preferred embodiments, this bioconversion is achieved by microorganisms. By maintaining a low  
15   concentration of the intermediate in a conversion medium, the intermediate's catabolite repressive and/or enzymatic inhibitive effects are altered (*e.g.*, reduced). The present invention also provides various levels of intermediate concentration, substrates, intermediates and steps of converting the intermediate to the desired end-product.

### Definitions

20       Unless defined otherwise herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Various references (See *e.g.*, Singleton, *et al.*, DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY, 2D ED., John Wiley and Sons, New York [1994]; and Hale and Marham, THE HARPER COLLINS DICTIONARY OF BIOLOGY, Harper Perennial, NY  
25   [1991]) provide general definitions of many of the terms used herein. Furthermore, all patents and publications, including all sequences disclosed within such patents and publications, referred to herein are expressly incorporated by reference.

Although any methods and materials similar or equivalent to those described herein find use in the practice of the present invention, preferred methods and materials are  
30   described herein. Numeric ranges are inclusive of the numbers defining the range. Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation, respectively. It is to be understood that this invention is not limited to the particular methodology, protocols, and reagents described, as these may vary.

35       The headings provided herein are not limitations of the various aspects or

embodiments of the invention which can be had by reference to the specification as a whole. Furthermore, the terms defined immediately below are more fully defined by reference to the Specification as a whole.

5           As used herein, the term "carbon substrate" refers to a material containing at least one carbon atom which can be enzymatically converted into an intermediate for subsequent conversion into the desired carbon end-product. Exemplary carbon substrates include, but are not limited to biomass, starches, dextrins and sugars.

          As used herein, "biomass" refers to cellulose- and/or starch-containing raw materials,  
10 including but not limited to wood chips, corn stover, rice, grasses, forages, perrie-grass, potatoes, tubers, roots, whole ground corn, cobs, grains, wheat, barley, rye, milo, brans, cereals, sugar-containing raw materials (e.g., molasses, fruit materials, sugar cane or sugar beets), wood, and plant residues. Indeed, it is not intended that the present invention be limited to any particular material used as biomass. In preferred embodiments of the present  
15 invention, the raw materials are starch-containing raw materials (e.g., cobs, whole ground corns, corns, grains, milo, and/or cereals, and mixtures thereof). In particularly preferred embodiments, the term refers to any starch-containing material originally obtained from any plant source.

          As used herein, "starch" refers to any starch-containing materials. In particular, the  
20 term refers to various plant-based materials, including but not limited to wheat, barley, potato, sweet potato, tapioca, corn, maize, cassava, milo, rye, and brans. Indeed, it is not intended that the present invention be limited to any particular type and/or source of starch. In general, the term refers to any material comprised of the complex polysaccharide carbohydrates of plants, comprised of amylose and amylopectin, with the formula  
25  $(C_6H_{10}O_5)_x$  wherein "x" can be any number.

          As used herein, "cellulose" refers to any cellulose-containing materials. In particular, the term refers to the polymer of glucose (or "cellobiose"), with the formula  $(C_6H_{10}O_5)_x$ , wherein "x" can be any number. Cellulose is the chief constituent of plant cell walls and is among the most abundant organic substances in nature. While there is a  $\beta$ -glucoside  
30 linkage in cellulose, there is an  $\alpha$ -glucoside linkage in starch. In combination with lignin, cellulose forms "lignocellulose."

          As used herein, "intermediate" refers to a compound that contains at least one carbon atom into which the carbon substrates are enzymatically converted. Exemplary intermediates include, but are not limited to pentoses (e.g., xylose, arabinose, lyxose,  
35 ribose, ribulose, xylulose); hexoses (e.g., glucose, allose, altrose, mannose, gulose, idose, galactose, talose, psicose, fructose, sorbose, and tagatose); and organic acids thereof.

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As used herein, the term "enzymatic conversion" refers to the modification of a carbon substrate to an intermediate or the modification of an intermediate to an end-product by contacting the substrate or intermediate with an enzyme. In some embodiments, contact is made by directly exposing the substrate or intermediate to the appropriate enzyme. In  
5 other embodiments, contacting comprises exposing the substrate or intermediate to an organism that expresses and/or excretes the enzyme, and/or metabolizes the desired substrate and/or intermediate to the desired intermediate and/or end-product, respectively.

As used herein, the term "starch hydrolyzing enzyme" refers to any enzyme that is capable of converting starch to the intermediate sugar (e.g., a hexose or pentose).

10 As used herein, "monosaccharide" refers to any compound having an empirical formula of  $(CH_2O)_n$ , wherein n is 3-7, and preferably 5-7. In some embodiments, the term refers to "simple sugars" that consist of a single polyhydroxy aldehyde or ketone unit. The term encompasses, but is not limited to such compounds as glucose, galactose, and fructose.

15 As used herein, "disaccharide" refers to any compound that comprises two covalently linked monosaccharide units. The term encompasses, but is not limited to such compounds as sucrose, lactose and maltose.

As used herein, "oligosaccharide" refers to any compound having 2 - 10 monosaccharide units joined in glycosidic linkages. In some preferred embodiments, the  
20 term refers to short chains of monosaccharide units joined together by covalent bonds.

As used herein, "polysaccharide" refers to any compound having multiple monosaccharide units joined in a linear or branched chain. In some preferred embodiments, the term refers to long chains with hundreds or thousands of monosaccharide units. Some polysaccharides, such as cellulose have linear chains, while others (e.g., glycogen) have  
25 branched chains. Among the most abundant polysaccharides are starch and cellulose, which consist of recurring glucose units (although these compounds differ in how the glucose units are linked).

As used herein, "culturing" refers to fermentative bioconversion of a carbon substrate to the desired end-product (typically within a reaction vessel). In particularly preferred  
30 embodiments, culturing involves the growth of microorganisms under suitable conditions for the production of the desired end-product(s).

As used herein, the term "saccharification" refers to converting a directly unusable polysaccharide to a useful sugar feed-stock for bioconversion or fermentative bioconversion.

As used herein, the term "fermentation" refers to the enzymatic and anaerobic  
35 breakdown of organic substances by microorganisms to produce simpler organic products.

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In preferred embodiments, fermentation refers to the utilization of carbohydrates by microorganisms (e.g., bacteria) involving an oxidation-reduction metabolic process that takes place under anaerobic conditions and in which an organic substrate serves as the final hydrogen acceptor (*i.e.*, rather than oxygen). Although fermentation occurs under anaerobic conditions, it is not intended that the term be solely limited to strict anaerobic conditions, as fermentation also occurs in the presence of oxygen.

As used herein, the terms "substantially all consumed" and "substantially all bioconverted" refer to the maintenance of a low level of intermediate in a conversion medium which adversely affects the enzymatic inhibition, oxygen transfer, yield, by-product minimization and/or catabolite repression effects of the intermediate (e.g., a hexose), upon the ability of the intermediate converting enzyme to convert the intermediate to the end-product or another intermediate and/or the ability of the substrate converting enzyme to convert the substrate to the intermediate.

As used herein, the terms "bioconversion" and "bioconverted" refer to contacting a microorganism with the carbon substrate or intermediate, under conditions such that the carbon substrate or intermediate is converted to the intermediate or desired end-product, respectively. In some embodiments, these terms are used to describe the production of another intervening intermediate in *in vitro* methods in which biocatalysts alone are used. In some preferred embodiments, the terms encompass metabolism by microorganisms and/or expression or secretion of enzyme(s) that achieve the desired conversion.

As used herein, the terms "conversion media" and "conversion medium" refer to the medium/media in which the enzymes and the carbon substrate, intermediate and end-products are in contact with one another. These terms include, but are not limited to fermentation media, organic and/or aqueous media dissolving or otherwise suspending the enzymes and the carbon substrate, intermediate and end-products. In some embodiments, the media are complex, while in other preferred embodiments, the media are defined.

As used herein, the term "end-product" refers to any carbon-source derived molecule product which is enzymatically converted from the intermediate. In particularly preferred embodiments, the methods of the present invention are used in order to produce a "desired end-product" (*i.e.*, the product that is intended to be produced through the use of these methods).

As used herein, "low concentration" refers to a concentration level of a compound that is less than that would result in the production of detrimental effects due to the presence of the compound. In particularly preferred embodiments, the term is used in reference to the concentration of a particular intermediate below which the detrimental effects of catabolite

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suppression and/or enzyme inhibition are observed. In some embodiments, the term refers to the concentration level of a particular intermediate above which triggers catabolite repression and/or enzymes inhibition by substrate and/or products.

As used herein, the phrase "maintained at a level below which triggers catabolite repression effects" refers to maintaining the concentration of an intermediate to below that level which triggers catabolite repression.

As used herein, the term "reduces catabolite repression" means conditions under which the effects of catabolite repression are produced. In preferred embodiments, the term refers to conditions in which the intermediate concentration is less than that threshold which triggers catabolite repressive effects.

As used herein, the term "reduces enzyme inhibition" means conditions under which the inhibition of an enzyme is reduced as compared to the inhibition of the enzyme under usual, standard conditions. In preferred embodiments of the present invention, the term refers to conditions in which the concentration of an intermediate, substrate and/or product of the enzyme reaction is less than that threshold which triggers enzyme inhibition.

As used herein, the term "substrate converting enzyme" refers to any enzyme that converts the substrate (e.g., granular starch) to an intermediate, (e.g., glucose). Substrate converting enzymes include, but are not limited to alpha-amylases, glucoamylases, pullulanases, starch hydrolyzing enzymes and various combinations thereof.

As used herein, the term "intermediate converting enzyme" refers to any enzyme that converts an intermediate (e.g., D-glucose, D-fructose, etc.), to the desired end-product. In preferred embodiments, this conversion is accomplished through hydrolysis, while in other embodiments, the conversion involves the metabolism of the intermediate to the end-product by a microorganism. However, it is not intended that the present invention be limited to any particular enzyme or means of conversion. Indeed, it is intended that any appropriate enzyme will find use in the various embodiments of the present invention.

As used herein, "yield" refers to the amount of end-product or intermediate produced using the methods of the present invention. In some preferred embodiments, the yield produced using the methods of the present invention is greater than that produced using methods known in the art. In some embodiments, the yield refers to the volume of the end-product or intermediate, while in other embodiments, the term is used in reference to the concentration of the end-product or intermediate in a composition.

As used herein, the term "oxygen transfer" refers to having sufficient dissolved oxygen in the bioconversion and/or fermentative bioconversion medium transferred from gas phase to a liquid medium such that it is not a rate limiting step.

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As used herein, "by-product formation" refers to the production of products that are not desired. In some preferred embodiments, the present invention provides methods that avoid or reduce the production of by-products, as compared to methods known in the art.

As used herein, the term "enzymatic inhibition" refers to loss of enzyme activity by  
5 either physical or biochemical effects on the enzyme. In some embodiments, inhibition results from the effects of the product formed by the enzyme activity, while in other embodiments, inhibition results from the action of the substrate or intermediate on the enzyme.

As used herein, "enzyme activity" refers to the action of an enzyme on its substrate.  
10 In some embodiments, the enzyme activity is quantitated using means to determine the conversion of the substrate to the intermediate, while in other embodiments, the conversion of the substrate to the end-product is determined, while in still further embodiments, the conversion of the intermediate to the end-product is determined.

As used herein, the term "enzyme unit" refers to the amount of enzyme which  
15 converts 1 micromole of substrate per minute to the substrate product at optimum assay conditions (unless otherwise noted). In some embodiments, commercially available enzymes (e.g., SPEZYME®, DISTALLASE®, OPTIMAX®; Genencor International) find use in the methods of the present invention.

As used herein, the term "glucoamylase unit" (GAU) is defined as the amount of  
20 enzyme required to produce one micromole of glucose per minute under assay conditions of 40° C. and pH 5.0.

As used herein, the term "glucose oxidase unit" (GOU) is defined as the amount of enzyme required to oxidize one micromole of D-glucose per minute under assay conditions of 25° C. and pH 7.0, to gluconic acid.

25 As used herein, the term "catalase units" (CU) is defined as the amount of enzyme required to decompose 1 micromole of hydrogen peroxide per minute under assay conditions of 25° C. and pH 7.0.

As used herein, one AG unit (GAU) is the amount of enzyme which splits one micromole of maltose per minute at 25° C. and pH 4.3. In some embodiments of the present  
30 invention, a commercially available liquid form of glucoamylase (OPTIDEX® L-400; Genencor International) with an activity of 400 GAU per ml is used.

As used herein, "carbon end-product" means any carbon product produced from the carbon intermediate, wherein the substrate contains at least one carbon atom (*i.e.*, a carbon substrate).

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As used herein, "carbon intermediate" refers to the carbon-containing compounds that are produced during the conversion of a carbon-containing substrate to a carbon end-product.

As used herein, "enzymatically controlled" means regulating the amount of carbon  
5 intermediate produced from the carbon substrate by altering the amount or activity of the enzyme used in the reaction.

As used herein, "microorganism" refers to any organism with cells that are typically considered to be microscopic, including such organisms as bacteria, fungi (yeasts and molds), rickettsia, and protozoa. It is not intended that the present invention be limited to  
10 any particular microorganism(s) or species of microorganism(s), as various microorganisms and microbial enzymes are suitable for use in the present invention. It is also not intended that the present invention be limited to wild-type microorganisms, as microorganisms and microbial enzymes produced using recombinant DNA technologies also find use in the present invention.

As used herein, "microbial enzyme" refers to any enzyme that is produced by a  
15 microorganism. As used herein, a "microbial intermediate-converting enzyme" is an enzyme that converts an intermediate to an end-product, while a "microbial substrate-converting enzyme" is an enzyme that converts a substrate to an intermediate or directly converts a substrate to an end-product (*i.e.*, there is not intermediate compound).

As used herein, "gluconic acid" refers to an oxidative product of glucose, wherein the  
20 C6 hydroxyl group of glucose is oxidized to a carboxylic acid group.

As used herein, the terms "gluconic acid producer" and "gluconic acid producing organism" refers to any organism or cell that is capable of producing gluconic acid through the use of a hexose or a pentose. In some embodiments, gluconic acid producing cells  
25 contain a cellulase as a substrate converting enzyme, and glucose oxidase and catalase for the conversion of the intermediates to gluconic acid.

As used herein, "glycerol producer" and "glycerol producing organism" refer to any organism or cell capable of producing glycerol. In some embodiments, glycerol producing organisms are aerobic bacteria, while in other embodiments, they are anaerobic bacteria. In  
30 still further embodiments, glycerol producing organisms include microorganisms such as fungi (*i.e.*, molds and yeast), algae and other suitable organisms.

As used herein, the terms "diol producer," "propanediol producer," "diol producing organism," and "propanediol producing organism" refer to any organism that is capable of producing 1,3-propanediol utilizing glycerol. Generally, diol producing cells contain either a  
35 diol dehydratase enzyme or a glycerol dehydratase enzyme.

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As used herein, the terms "lactate producer," and "lactate producing organism," and "lactate producing microorganism" refer to any organism or cell that is capable of producing lactate by utilizing a hexose or a pentose. In some embodiments, the lactate producers are members of the genera *Lactobacillus* or *Zymomonas*, while in other embodiments, they  
5 organisms are fungi.

As used herein, the terms "ethanol producer" and "ethanol producing organism" refer to any organism or cell that is capable of producing ethanol from a hexose or a pentose. Generally, ethanol producing cells contain an alcohol dehydrogenase and pyruvate decarboxylase.

10 As used herein, the term "ascorbic acid intermediate producer" and "ascorbic acid intermediate producing organism" refers to any organism or cell that is capable of producing an ascorbic acid intermediate from a hexose or a pentose. Generally, ethanol producing cells contain a glucose dehydrogenase, gluconic acid dehydrogenase, 2,5-diketo-D-gluconate reductase, 2-keto-D-gluconate reductase, 2-keto-reductase, 5-keto reductase,  
15 glucokinase, glucono kinase, ribulose-5-phosphate epimerase, transketolase, transaldolase, hexokinase, 2,5-DKG reductase, and/or idonate dehydrogenase, depending upon the specific ascorbic acid intermediate desired.

As used herein, the term "ascorbic acid intermediate" refers to any of the following compounds: D-gluconate, 2-keto-D-gluconate (2KDG), 2,5-diketo-D-gluconate (2,5-DKG or 5 DKG), 2-keto-L-gulonate (2KLG or KLG), L-idonic acid (IA), erythorbic acid (EA), and ascorbic acid (ASA).  
20

As used herein, "citric acid" refers to having the formula  $C_6H_8O_7$ , commonly found in citrus fruits, beets, cranberries and other acid fruits. The term refers to citric acid from any source, whether natural or synthetic, as well as salts and any other form of the acids.

25 As used herein, "succinic acid" refers to the acid having the formula  $C_4H_6O_4$ , which is commonly found in amber, algae, lichens, sugar cane, beets and other plants. This acid is also formed during the fermentation of sugar, tartrates, malates, and other substances by various molds, yeasts and bacteria. The term refers to succinic acid from any source, whether natural or synthetic, as well as acid and neutral salts and esters, and any other form  
30 of the acid.

As used herein, "amino acid" refers to any of naturally-occurring amino acids, as well as any synthetic amino acids, including amino acid derivatives.

As used herein, "antimicrobial" refers to any compound that kills or inhibits the growth of microorganisms (including but not limited to antibacterial compounds).

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As used herein, the term "linked culture" refers to a fermentation system that employs at least two cell cultures, in which the cultures are added sequentially. In most embodiments of linked systems, a primary culture or a set of primary cultures is grown under optimal fermentation conditions for the production of a desired intermediate (*i.e.*, the intermediate is released into the culture media to produce a "conditioned medium").  
Following the fermentation of the primary culture, the conditioned medium is then exposed to the secondary culture(s). The secondary cultures then convert the intermediate in the conditioned media to the desired end-product. In some embodiments of the present invention, the primary cultures are typically glycerol producers and the secondary cultures are 1,3-propanediol producers.

As used herein, "mixed culture" refers to the presence of any combination of microbial species in a culture. In some preferred embodiments, the mixed culture is grown in a reaction vessel under conditions such that the interaction of the individual metabolic processes of the combined organisms results in a product which neither individual organism is capable of producing. It is not intended that the present invention be limited to mixed cultures comprising a particular number of microbial species.

As used herein, "conditioned media" refers to any fermentation media suitable for the growth of microorganisms that has been supplemented by organic by-products of microbial growth. In preferred embodiments of the present invention, conditioned media are produced during fermentation of linked cultures wherein glycerol producing cells secrete glycerol into the fermentation media for subsequent conversion to 1,3-propanediol.

As used herein, "oxygen uptake rate" ("OUR") refers to the determination of the specific consumption of oxygen within a reaction vessel. Oxygen consumption can be determined using various on-line measurements known in the art. In one embodiment, the OUR (mmol/(liter\*hour)) is determined by the following formula: ((Airflow (standing liters per minute) / Fermentation weight (weight of the fermentation broth in kilograms)) X supply O<sub>2</sub> X broth density X (a constant to correct for airflow calibration at 21.1 C instead of standard 20.0 C)) minus ([airflow /fermentation weight] x [offgas O<sub>2</sub>/offgas N<sub>2</sub>] X supply N<sub>2</sub> X broth density X constant ).

As used herein, "carbon evolution rate" ("CER") refers to the determination of how much CO<sub>2</sub> is produced within a reaction vessel during fermentation. Usually, since no CO<sub>2</sub> is initially or subsequently provided to the reaction vessel, any CO<sub>2</sub> is assumed to be produced by the fermentation process occurring within the reaction vessel. "Off-gas CO<sub>2</sub>" refers to the amount of CO<sub>2</sub> measured within a reaction vessel, usually by mass spectroscopic methods known in the art.

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As used herein, the term "enhanced" refers to improved production of proteins of interest. In preferred embodiments, the present invention provides enhanced (*i.e.*, improved) production and secretion of a protein of interest. In these embodiments, the "enhanced" production is improved as compared to the normal levels of production by the host (*e.g.*, wild-type cells). Thus, for heterologous proteins, basically any expression is enhanced, as the cells normally do not produce the protein.

As used herein, the terms "isolated" and "purified" refer to a nucleic acid or amino acid that is removed from at least one component with which it is naturally associated.

As used herein, the term "heterologous protein" refers to a protein or polypeptide that does not naturally occur in a host cell. In alternate embodiments, the protein is a commercially important industrial protein or peptide. It is intended that the term encompass protein that are encoded by naturally occurring genes, mutated genes, and/or synthetic genes.

15

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention provides means for the production of desired end-products of *in vitro* and/or *in vivo* bioconversion of biomass-based feed stock substrates, including but not limited to such materials as starch and cellulose. In particularly preferred embodiments, the methods of the present invention do not require gelatinization and/or liquefaction of the substrate.

The methods of the present invention provide means for dramatic improvements in the process for directly converting a commonly available carbon substrate such as biomass and/or starch into an intermediate, particularly intermediates that are readily convertible into a multitude of desired end-products, such as primary metabolites (*e.g.* ascorbic acid intermediates, lactic acid, succinic acid, or amino acids), alcohols (*e.g.*, ethanol, propanol, and or 1,3 propanediol), and enzymes or secondary metabolites such as antimicrobials.

In some particularly preferred embodiments, the present invention provides means for dramatic improvements in processes for directly converting granular starch into glucose, an intermediate readily convertible into a multitude of desired end-products, such as primary metabolites (*e.g.* ascorbic acid intermediates, lactic acid, succinic acid, or amino acids), alcohols (*e.g.*, ethanol, propanol, and or 1,3 propanediol), and enzymes or secondary metabolites such as antimicrobials.

In alternative embodiments, the present invention provides means for dramatic improvements in the process for converting starch or cellulose into glucose, which in turn is

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converted into the desired end-product. By maintaining the presence of the intermediate at a low concentration within the conversion media, overall efficiency of the production is improved. In some embodiments, enzymatic inhibition and/or catabolite repression, oxygen uptake demand, and/or by-product formation are reduced.

5 In some preferred embodiments, the maintenance of minimal intermediate concentrations is achieved by maintaining the concentration of the intermediate at a low concentration. In one embodiment, the concentration of the intermediate is less than or equal to 0.25% by weight volume of the medium (*e.g.*, 0.25% to 0.00001% by weight volume). In other embodiments, the concentration of the intermediate is less than or equal  
10 to 0.20%, 0.10%, 0.05%, or 0.01% by weight volume (*e.g.*, 0.20% to 0.00001%, 0.10% to 0.00001%, 0.05% to 0.00001%, 0.01% to 0.00001%, respectively). Alternatively, the intermediate concentration is maintained at less than or equal to a concentration of 2.0  $\mu$ molar in the conversion media. In another embodiment, the concentration is maintained at less than or equal to 1.0  $\mu$ molar. In still another embodiment, the concentration of the  
15 intermediate is maintained at a concentration of less than or equal to 0.75  $\mu$ molar. In any event, maintaining a low concentration means maintaining the concentration of the intermediate below the threshold that results in enzyme inhibition (*i.e.*, enzyme inhibitive effects), catabolite repression (*i.e.*, catabolite repressive effects).

In further embodiments, the maintenance of a minimal concentration is achieved by  
20 maintaining the rate of conversion of the substrate to the intermediate at less than or equal to the rate of conversion of the intermediate to the end-product. While it is recognized that the conversion of the substrate to the intermediate is necessarily rate limiting for the conversion of the intermediate to the end-product, by providing sufficient intermediate converting enzymes for the conversion of substantially all of the intermediate produced by  
25 the first enzymatic conversion from the carbon substrate, substantially all of the intermediate is converted to the end-product as fast as it is converted from the starting substrate to minimize the presence of the intermediate in the conversion medium. Exemplary methods of providing such excessive intermediate conversion include providing an excess of intermediate converting enzyme, increasing the enzyme activity of the intermediate  
30 converting enzyme, and/or decreasing the activity of the substrate converting enzyme to convert the intermediate to end-product as quickly as it is converted from the substrate. As the actual rate of conversion is contemplated to vary with the specific end product produced, some variation in the amount and experimentation in determining the amount are contemplated. However guidelines for making these determinations are provided herein.

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In some embodiments of the present invention, the conversion or consumption rate of the intermediate was determined by the calculating the amount of organism present in the mixed media, taking into consideration the other physical parameters of the mixed media, and multiplying that amount by the generally known conversion rate. This provides a rate of conversion of the intermediate, (e.g., glucose), to the end-product. In some embodiments, this conversion of the intermediate to the desired end product is by conversion or bioconversion of the intermediate to the end-product by a naturally occurring organism or one mutated to provide such bioconversion. Another embodiment of the conversion from intermediate to end product involves the use of an enzymatic conversion by a known enzyme to the desired end-product using known enzymatic conversion methods. For example, in some embodiments, the conversion of glucose to a desired end product (e.g., propanediol, succinic acid, gluconic acid, lactic acid, amino acids, antimicrobials, ethanol, ascorbic acid intermediates and/or ascorbic acid) is accomplished by the addition of an amount of an enzyme known to convert glucose to the specified end product desired.

Once the conversion rate of the intermediate to the desired end product is determined, the limit of the conversion of the carbon substrate to the intermediate can be determined in the same manner. By calculating the upper limit of the intermediate to end product conversion, the conversion rate of the carbon substrate to intermediate can be determined, the main consideration being that the intermediate concentration levels in the conversion media are maintained at a sufficiently low level to adversely effect the normally catabolite repressive/enzymatic inhibitory effects of the intermediate. In one embodiment, this is accomplished by maintaining the conversion rate of the intermediate to the end product in excess or equal to the rate of conversion of the carbon substrate to the intermediate. Thus, the present invention provides means for increasing the conversion rate to the end product, as well as means for restricting the conversion of the carbon substrate to the intermediate.

Another method for determining whether the rate of conversion of the intermediate to the end product is greater than or equal to the production of the intermediate from the carbon substrate is to measure the weight percentage of the intermediate in a reaction vessel. The amount of the intermediate present in a reaction vessel can be determined by various known methods, including, but not limited to direct or indirect measurement of the amount of intermediate present in a reaction vessel. Direct measurement can be by periodic assays of the contents within a reaction vessel, using assays known to identify the amount of intermediate and or end-product in the vessel. In addition, direct measurement

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of the amounts of intermediates within a reaction vessel include on-line gas, liquid and/or high performance liquid chromatography methodologies known in the art

Indirect measurement of the levels of intermediate or end-products produced can be assessed by the measurement of oxygen uptake or carbon dioxide production, using  
5 methods known in the art (e.g., by determining the oxygen uptake rate and/or the carbon evolution rate).

### Substrates

The substrates of the present invention are carbon-based compounds that can be  
10 converted enzymatically to intermediate compounds. Suitable substrates include, but are not limited to processed materials that contain constituents which can be converted into sugars (e.g., cellulosic biomass, glycogen, starch and various forms thereof, such as corn starch, wheat starch, corn solids and wheat solids). During the development of the present invention good results were obtained with corn starch and wheat starch, although other  
15 sources, including starches from grains and tubers (e.g., sweet potato, potato, rice and cassava starch) also find use with the present invention. Various starches are commercially available. For example, corn starches are available from Cerestar, Sigma, and Katayama Chemical Industry Co. (Japan); wheat starches are available from Sigma; sweet potato starch is available from Wako Pure Chemical Industry Co. (Japan); and potato starch is  
20 available from Nakari Chemical Pharmaceutical Co. (Japan). A particularly useful carbon substrate is corn starch. In some embodiments of the present invention, granular starch is used in a slurry having a percentage of starch between about 20% and about 35%. Preferably, the starch is in a concentration between about 10% and about 35%. In some particularly preferred embodiments, the range for percent starch is between 30% and 32%.  
25 In addition to raw granular starch, other carbon substrate sources find use in the present invention include, but are not limited to biomass, polysaccharides, and other carbon based materials capable of being converted enzymatically to an intermediate.

Fermentable sugars can be obtained from a wide variety of sources, including lignocellulosic material. Lignocellulose material can be obtained from lignocellulosic waste  
30 products (e.g., plant residues and waste paper). Examples of suitable plant residues include but are not limited to any plant material such as stems, leaves, hulls, husks, cobs and the like, as well as corn stover, bagasses, wood, wood chips, wood pulp, and sawdust. Examples of paper waste include but are not limited to discarded paper of any type (e.g., photocopy paper, computer printer paper, notebook paper, notepad paper, typewriter paper,  
35 and the like), as well as newspapers, magazines, cardboard, and paper-based packaging

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materials. The conditions for converting sugars to ethanol are known in the art. Generally, the temperature is between about 25 ° C. and 35 ° C (e.g., between 25° and 35°, and more particularly at 30° C). Useful pH ranges for the conversion medium are provided between about 4.0 and 6.0, between 4.5 and 6.0, and between pH 5.5 and 5.8. However, it is not  
5 intended that the present invention be limited to any particular temperature and/or pH conditions as these conditions are dependent upon the substrate(s), enzyme(s), intermediate(s), and/or end-product(s) involved.

### Enzymes

10 In some preferred embodiments of the present invention, enzymes that are substrate converting enzymes (*i.e.*, enzymes that are able to first convert the carbon substrate into the carbon intermediate), and intermediate converting enzymes (*i.e.*, enzymes that are able to convert the resulting intermediate into an intervening intermediate and/or the desired end-product) both find use in the present invention. Enzymes that find use in some  
15 embodiments of the present invention to convert a carbon substrate to an intermediate include, but are not limited to alpha-amylase, glucoamylase, starch hydrolyzing glucoamylase, and pullulanases. Enzymes that find use in the conversion of an intermediate to an end-product depend largely on the actual desired end-product. For example enzymes useful for the conversion of a sugar to 1,3-propanediol include, but are  
20 not limited to enzymes produced by *E. coli* and other microorganisms. For example enzymes useful for the conversion of a sugar to lactic acid include, but are not limited to those produced by *Lactobacillus* and *Zymomonas*. Enzymes useful for the conversion of a sugar to ethanol include, but are not limited to alcohol dehydrogenase and pyruvate decarboxylase. Enzymes useful for the conversion of a sugar to ascorbic acid intermediates  
25 include, but are not limited to glucose dehydrogenase, gluconic acid dehydrogenase, 2,5-diketo-D-gluconate reductase, and various other enzymes. Enzymes useful for the conversion of a sugar to gluconic acid include, but are not limited to glucose oxidase and catalase.

In some preferred embodiments, the alpha-amylase used in some methods of the  
30 present invention is generally an enzyme which effects random cleavage of alpha-(1-4) glucosidic linkages in starch. In most embodiments, the alpha-amylase is chosen from among the microbial enzymes having an E. C. number E. C. 3.2.1.1 and in particular E. C. 3.2.1.1-3. In some preferred embodiments, the alpha-amylase is a thermostable bacterial alpha-amylase. In most particularly preferred embodiments, the alpha-amylase is obtained  
35 or derived from *Bacillus* species. Indeed, during the development of the present invention

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good results were obtained using the SPEZYME® alpha-amylase obtained from *Bacillus licheniformis* (Genencor). In other embodiments, black-koji amylase described in methods for alcoholic fermentation from starch such as corn and cassava without precooking (Ueda *et al.*, J. Ferment. Technol., 50:237-242 [1980]; and Ueda *et al.*, J. Ferment. Technol., 58:237-242 [1980]) find use in the present invention.

As understood by those in the art, the quantity of alpha-amylase used in the methods of the present invention will depend on the enzymatic activity of the alpha-amylase and the rate of conversion of the generated glucose by the end-product converter. For example, generally an amount between 0.01 and 1.0 kg of SPEZYME® FRED (Genencor) is added to one metric ton of starch. In some embodiments, the enzyme is added in an amount between 0.4 to 0.6 kg, while in other embodiments, it is added in an amount between 0.5 and 0.6 kg of SPEZYME® FRED/metric ton of starch..

In preferred embodiments of the present invention, the glucoamylase is an enzyme which removes successive glucose units from the non-reducing ends of starch. The enzyme can hydrolyze both the linear and branched glucosidic linkages of starch, amylose and amylopectin. In most embodiments, the glucoamylase used in the methods of the present invention are microbial enzymes. In some preferred embodiments, the glucoamylase is a thermostable fungal glucoamylase, such as the *Aspergillus* glucoamylase. Indeed, during the development of the present invention, good results were obtained using the DISTALLASE® glucoamylase derived from *Aspergillus niger* (Genencor). Glucoamylase preparations from *Aspergillus niger* have also been used without the use of precooking (See, Ueda *et al.*, Biotechnol. Bioeng., 23:291[1981]). Three glucoamylases have been selectively separated from *Aspergillus awamori* var. *kawachi* for use in hydrolyzing starch (See, Hayashida, Agr. Biol. Chem., 39:2093-2099 [1973]). Alcoholic fermentation of sweet potato by *Endomycopsis fibuligoeu* glucoamylase without cooking has also been described (Saha *et al.*, Biotechnol. Bioeng., 25:1181-1186 [1983]). Another enzyme that finds use in the present invention is glucoamylase (EC 3.2.1.3), an enzyme that hydrolyzes the alpha-1,4-glucoside chain progressively from the non-reducing terminal end. This enzyme also hydrolyzes the alpha-1,6-glucoside chain. Glucoamylase is secreted from fungi of the genera *Aspergillus*, *Rhizopus* and *Mucor* also find use in the methods of the present invention. These enzymes further find use in glucose production and quantitative determination of glycogen and starch. Glucoamylase preparations obtained from *E. fibuligera* (IFO 0111) have been used to contact raw sweet potato starch for alcoholic fermentation (See, Saha *et al.*, Biotechnol. Bioeng., 25:1181-1186 [1983]). One of this enzyme's major applications is as a saccharifying agent in the production of ethyl alcohol

from starchy materials. However, as with the other glucoamylases described herein, this enzyme also finds use in the methods of the present invention.

Additional glucoamylases that find use in the methods of the present invention include those obtained from the genera *Rhizopus* and *Humicola*, which are characterized as having particularly high productivity and enzymatic activity. Furthermore, in comparison with the glucoamylase derived from other organisms, the *Rhizopus*-derived glucoamylase exhibits a strong action on starch and its enzymological and chemical properties including optimum pH are particularly suitable for the saccharification of cereal starch. Because of these features, the *Rhizopus*-derived glucoamylase is considered to be best suited for alcohol production using non-cooked or low-temperature cooked starch (See, U.S. Pat. No. 4,514,496 and 4,092,434). It has been noted that upon the incubation of raw corn starch with *Rhizopus* glucoamylase, was used in conjunction with *Rhizopus* alpha amylase, the starch degradation by glucoamylase was accelerated. While it is not intended that the present invention be limited to any particular mechanism or theory, it is believed that *Rhizopus* glucoamylase has a stronger degradation activity than *Aspergillus niger* glucoamylase preparations which also contain  $\alpha$ -amylase (See, Yamamoto *et al.*, Denpun Kagaku, 37:129-136 [1990]). One commercial preparation that finds use in the present invention is the glucoamylase preparation derived from the Koji culture of a strain of *Rhizopus niveus* available from Shin Nippo Chemical Co., Ltd. Another commercial preparation that finds use in the present invention is the commercial starch hydrolyzing composition M1 is available from Biocon India, of Bangalore, India.

As understood by those in the art, the quantity of glucoamylase used in the methods of the present invention depends on the enzymatic activity of the glucoamylase (e.g., DISTILLASE® L-400). Generally, an amount between 0.001 and 2.0 ml of a solution of the glucoamylase is added to 450 gm of a slurry adjusted to 20-35% dry solids, the slurry being the liquefied mash during the saccharification and/or in the hydrolyzed starch and sugars during the fermentation. In some embodiments, the glucoamylase is added in an amount between 0.005 and 1.5 ml of such a solution. In some preferred embodiments, the enzyme is added at an amount between 0.01 and 1.0 ml of such a solution.

As indicated above, pullulanases also find use in the methods of the present invention. These enzymes hydrolyze  $\alpha$ -1,6-glucosidic bonds. Thus, during the saccharification of the liquefied starch, pullulanases remove successive glucose units from the non-reducing ends of the starch. This enzyme is capable of hydrolyzing both the linear and branched glucosidic linkages of starch, amylose and amylopectin.

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Additional enzymes that find use in the present invention include starch hydrolyzing (RSH) enzymes, including *Humicola* RSH glucoamylase enzyme preparation (See, U.S. Patent No. 4,618,579). This *Humicola* RSH enzyme preparation exhibits maximum activity within the pH range of 5.0 to 7.0 and particularly in the range of 5.5 to 6.0. In addition, this enzyme preparation exhibits maximum activity in the temperature range of 50° C. to 60° C. Thus, in each of the steps of the present invention in which this enzyme is used, the enzymatic solubilization of starch is preferably carried out within these pH and temperature ranges.

In some embodiments, *Humicola* RSH enzyme preparations obtained from the fungal organism strain *Humicola grisea* var. *thermoidea* find use in the methods of the present invention. In some particularly preferred embodiments, these *Humicola* RSH enzymes are selected from the group consisting of ATCC (American Type Culture Collection) 16453, NRRL (USDA Northern Regional Research Laboratory) 15219, NRRL 15220, NRRL 15221, NRRL 15222, NRRL 15223, NRRL 15224, and NRRL 15225, as well as genetically altered strains derived from these enzymes.

Additional RSH glucoamylases that find use in the methods of the present invention include *Rhizopus* RSH glucoamylase enzyme preparations. In some embodiments, the enzyme obtained from the Koji strain of *Rhizopus niveus* available from Shin Nihon Chemical Co., Ltd., Ahjyo, Japan, under the tradename "CU CONC" is used. Another useful enzyme preparation is a commercial digestive from *Rhizopus* available from Amano Pharmaceutical under the tradename "GLUCZYME" (See, Takahashi *et al.*, J. Biochem., 98:663-671 [1985]). Additional enzymes include three forms of glucoamylase (EC 3.2.1.3) of a *Rhizopus* sp., namely "Gluc1" (MW 74,000), "Gluc2" (MW 58,600) and "Gluc 3" (MW 61,400). Gluc1 was found to be 22-25 times more effective than Gluc2 or Gluc3. Thus, although Gluc2 and Gluc3 find use in the present invention, because Gluc1 tightly binds to starch and has an optimum pH of 4.5, Gluc1 finds particular use in the present invention. An additional RSH glucoamylase enzyme preparation for use in the present invention includes enzyme preparations sold under the designation "M1," available from Biocon India, Ltd., Bangalore, India (M1 is a multifaceted enzyme composition or mixture).

As noted above, in most embodiments, *Humicola* RSH glucoamylase enzyme preparations contain glucoamylase activity as well as a potentiating factor which solubilizes starch. The relative proportions of potentiating factor and glucoamylase activity in other RSH enzyme preparations may vary somewhat. However, with RSH glucoamylase enzyme preparations that find use in the present invention, there is usually ample potentiating factor

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produced along with the glucoamylase fraction. Accordingly, the activity of the RSH glucoamylase enzyme preparations is defined in terms of their glucoamylase activity.

In addition to the use of enzymatic compositions containing the above described hydrolyzing enzymes, the present invention provides methods in which a microorganism is exposed to a substrate and uses the substrate to produce the desired end-product. Thus, in some embodiments, contacting the substrate or intermediate with a fungal, bacterial or other microorganism that produces the desired end-product is used to convert the substrate or intermediate to the desired intermediate or end-product. For example, *Lactobacillus amylovorus* (ATCC 33621) is a lactic acid producing bacteria isolated from cattle manure corn enrichments (See, Nkamura, Int. J. Syst. Bacteriol., 31:56-63 [1981]). This strain produces an extracellular amylase which enables it to hydrolyze liquefied (soluble) starch to glucose, which can then be fermented to lactic acid. (See, Xiaodong *et al.*, Biotechnol. Lett., 19:841-843 [1997]). *E. coli* produces 1,3-propanediol and succinic acid, which can be contacted with glucose to produce glycerol and 1,3-propanediol.

Indeed, commercially available alpha-amylases and glucoamylases find use in the methods of the present invention in economically realistic enzyme concentrations. Although commonly used fermentation conditions do not utilize optimum temperatures, the pH conditions for fermentation do correspond closely to the optimum pH for commercially available saccharification enzymes (*i.e.*, the glucoamylases). In some embodiments of the present invention, complete saccharification to glucose is favored by the gradual solubilization of granular starch. Presumably, the enzyme is always exposed to low concentrations of dextrin. In addition, the removal of glucose throughout the fermentation maintains a low glucose content in the fermentation medium. Thus, glucoamylase is exposed to low concentration of glucose. In consequence, the glucoamylase is used so effectively that economically feasible dosage levels of glucoamylase (GAU) are suitable for use in the methods of the present invention (*i.e.*, glucoamylase dosage of 0.05-10.0 GAU/g of starch; and preferably 0.2-2.0 GAU/g starch).

The dosages provided above for glucoamylase only approximate the effective concentration of the enzymatic saccharification activity in the fermentation broth, as an additional proportion of the saccharification activity is contributed by the alpha-amylase. Although it is not intended that the present invention be limited to any particular mechanism or theory, it is believed that the alpha-amylase further widens the holes bored by glucoamylase on starch granules (See, Yamamoto *et al.*, *supra*). Typically, the use of commercially available alpha-amylases results in the production of significant amounts of sugars, such as glucose and maltose.

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It is contemplated that addition of the alpha-amylase from *Aspergillus oryzae* (e.g., CLARASE® L (Genencor International Inc.) to wort will find use in the brewing industry. This particular enzyme saccharifies dextrans to maltotriose and maltose. Thus, although the purpose of the alpha-amylase is to liquefy starch, its saccharification propensity also make  
5 the alpha-amylase a portion of the saccharifying enzyme content.

Furthermore, some commercially available glucoamylases contain some alpha-amylase activity. Thus, it is possible (albeit usually not practical) to ferment particulate starch in the presence solely of glucoamylase. However, it is not intended that such embodiments be excluded from the present invention.

10 In most embodiments of the methods of the present invention, an effective amount of alpha-amylase is added to a slurry of particulate starch. Those of skill in the art understand that in addition to the uncertain amount of alpha-amylase activity contributed by glucoamylase, the effective activity of the alpha-amylase may be quite different from the unit activity values given by the supplier. The activity of alpha-amylase is pH dependent, and  
15 may be different at the pH range selected for the fermentation (i.e., as compared with the test conditions employed by the suppliers for their reported unit activity values). Thus, some preliminary experiments are contemplated as being sometimes necessary in order establish the most effective dosages for alpha-amylases, including those not explicitly described herein, but find use in the methods of the present invention.

20 In some most preferred embodiments, the alpha-amylase dosage range for fungal alpha-amylases is from 0.02 SKBU/g (Fungal Alpha Amylase Units) to 2.0 SKBU/g of starch, although in some particularly preferred embodiments, the range is 0.05-0.6 SKBU/g. One "SKBU" is as known in the art (See, Cerial Chem., 16:712-723 [1939]). In most embodiments utilizing *Bacillus* alpha-amylases, the range is 0.01 LU/g to 0.6 LU/g,  
25 preferably 0.05 to 0.15 LU/g. It is contemplated that the uncertainty as to the real activity of both the glucoamylase and the alpha-amylase in the fermenting slurry will require some preliminary investigation into the practice of some embodiments. Optimization considerations include the fact that increasing the alpha-amylase dosage with a constant glucoamylase content, increases the fermentation rate. In addition, increasing the  
30 glucoamylase dosage with a constant alpha-amylase content increases the fermentation rate. Holding the dosage of enzyme constant and/or increasing the starch content in the slurry also increase the fermentation rate. Indeed, it is contemplated that in some embodiments, the optimum alpha-amylase dosage well exceeds dosages heretofore recommended for liquefying starch; the optimum glucoamylase may well exceed dosages  
35 recommended for saccharifying syrups. However, enzyme dosage levels should not be

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confused with enzyme usage. Substantial proportions of the enzymes dosed into the starch slurry are available for recovery from the fermentation broth for use anew to ferment granular starch.

A further consideration arising from employment of the enzymes at fermentation  
5 temperatures is that although the enzymes exhibit low relative activity (e.g., activity of the alpha-amylase from *B. licheniformis* at fermentation temperatures is not more than about 25% of maximum activity), the low relative activity is counterbalanced by the extended duration of the 48-120 hours of fermentation, and by the extended half-life of enzymes that have not been subjected to elevated temperatures. Indeed, it has been determined that  
10 more than 90% of the enzymes activity remains after 72 hours of fermentation.

The alpha-amylase of *B. licheniformis* (SPEZYME® AA and SPEZYME® FRED enzymes; Genencor International Inc.) is sufficiently stable to withstand brief exposures to still pot temperatures. Thus, recycle of stillage can be used as a way to recycle alpha-amylase. However, recovery of enzyme in recycled stillage requires care, in avoiding  
15 subjecting the fermentation broth to ethanol stripping temperatures that deactivate the enzyme(s). For example, the alcohol may be vacuum stripped from the fermentation broth and such stillage recycled to recover the enzymes suitable for use in subsequent reactions.

However, as earlier described, some RSHs glucoamylases (e.g., the enzyme obtained from *Rhizopus*) are available that convert starch to glucose at non-cooking  
20 temperatures, reducing the need for exposing the enzymatic composition to still pot temperatures. This reduces the energy costs of converting the carbon substrate to the desired end-product, thereby reducing the overall costs of manufacturing. Thus, these enzymes find particular use in the methods of the present invention.

In preferred embodiments of the present invention, once the carbon source is  
25 enzymatically converted to the intermediate, it is converted into the desired end-product by the appropriate methodology. Conversion is accomplished via any suitable method (e.g., enzymatic or chemical). In one preferred embodiment, conversion is accomplished by bioconversion of the intermediate by contacting the intermediate with a microorganism. In alternate preferred embodiments, the respective substrate-converting enzyme and the  
30 intermediate-converting enzyme are placed in direct contact with the substrate and/or intermediate. In some embodiments, the enzyme(s) are provided as isolated, purified or concentrated preparations.

In further embodiments, the substrate and/or intermediate are placed in direct contact with a microorganism (e.g., bacterium or fungus) that secretes or metabolizes the  
35 respective substrate or intermediate. Thus, the present invention provides means for the

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bioconversion of a substrate to an end-product. In some embodiments, at least one intermediate compound is produced during this conversion process.

In some embodiments, microorganisms that are genetically modified to express enzymes not normally produced by the wild-type organism are utilized. In some particularly preferred embodiments, the organisms are modified to overexpress enzymes that are normally produced by the wild-type organism.

The desired end-product can be any product that may be produced by the enzymatic conversion of the substrate to the end-product. In some preferred embodiments, the substrate is first converted to at least one intermediate and then converted from the intermediate to an end-product. For example, hexoses can be bioconverted into numerous products, such as ascorbic acid intermediates, ethanol, 1,3-propanediol, and gluconic acid. Ascorbic acid intermediates include but are not limited to 2,5-diketogluconate, 2 KLG (2-keto-L-gluconate), and 5-KDG (5-keto-D-gluconate). Gluconate can be converted from glucose by contacting glucose with glucose dehydrogenase (GDH). In addition, gluconate itself can be converted to 2-KDG (2-keto-D-gluconate) by contacting gluconate with GDH. Furthermore, 2-KDG can be converted to 2,5-DKG by contacting 2-KDG with 2-KDGH. Gluconate can also be converted to 2-KDG by contacting gluconate with 2KR. Glucose can also be converted to 1,3-propanediol by contacting glucose with *E. coli*. In addition, glucose can be converted to succinic acid by contacting glucose with *E. coli*. Additional embodiments, as described herein are also provided by the present invention.

In some embodiments in which glucose is an intermediate, it is converted to ethanol by contacting glucose with an ethanologenic microorganism. In contacting the intermediate with an intermediate converting enzyme, it is contemplated that isolated and/purified enzymes are placed into contact with the intermediate. In yet another embodiment, the intermediate is contacted with bioconverting agents such as bacteria, fungi or other organism that takes in the intermediate and produces the desired end-product. In some embodiments, the organism is wild-type, while in other embodiments it is mutated.

Preferred examples of ethanologenic microorganisms include ethanologenic bacteria expressing alcohol dehydrogenase and pyruvate decarboxylase, such as can be obtained with or from *Zymomonas mobilis* (See e.g., U.S. Pat. Nos. 5,028,539, 5,000,000, 5,424,202, 5,487,989, 5,482,846, 5,554,520, 5,514,583, and copending applications having U.S. Ser. No. 08/363,868 filed on Dec. 27, 1994, U.S. Ser. No. 08/475,925 filed on Jun. 7, 1995, and U.S. Ser. No. 08/218,914 filed on Mar. 28, 1994).

In additional embodiments, the ethanologenic microorganism expresses xylose reductase and xylitol dehydrogenase, enzymes that convert xylose to xylulose. In further

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embodiments, xylose isomerase is used to convert xylose to xylulose. In additional  
embodiments, the ethanologenic microorganism also expresses xylulokinase, an enzyme  
that catalyzes the conversion of xylulose to xylulose-5-phosphate. Additional enzymes  
involved in the completion of the pathway include transaldolase and transketolase. These  
5 enzymes can be obtained or derived from *Escherichia coli*, *Klebsiella oxytoca* and *Erwinia*  
species (See e.g., U.S. Pat. No. 5,514,583).

In some particularly preferred embodiments, a microorganism capable of fermenting  
both pentoses and hexoses to ethanol are utilized. For example in some embodiments, a  
recombinant organism which inherently possesses one set of enzymes and which is  
10 genetically engineered to contain a complementing set of enzymes is used (See e.g., U.S.  
Pat. Nos. 5,000,000, 5,028,539, 5,424,202, 5,482,846, 5,514,583, and WO 95/13362). In  
some embodiments, particularly preferred microorganisms include *Klebsiella oxytoca* P2  
and *E. coli* KO11.

In some embodiments, supplements are added to the nutrient medium (i.e., the  
15 culture medium), including, but not limited to vitamins, macronutrients, and micronutrients.  
Vitamins include, but are not limited to choline chloride, nicotinic acid, thiamine HCl,  
cyanocobalamin, p-aminobenzoic acid, biotin, calcium pantothenate, folic acid,  
pyridoxine.HCl, and riboflavin. Macronutrients include, but are not limited to  $(\text{NH}_4)_2\text{SO}_4$ ,  
 $\text{K}_2\text{HPO}_4$ , NaCl, and  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ . Micronutrients include, but are not limited to  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ,  
20  $\text{ZnCl}_2 \cdot 4\text{H}_2\text{O}$ ,  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , molybdic acid (tech),  $\text{CuCl}_3 \cdot 2\text{H}_2\text{O}$ ,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , and  $\text{H}_3\text{BO}_3$ .

### Media and Carbon Substrates

The conversion media in the present invention must contain suitable carbon  
substrates. Suitable carbon substrates include, but are not limited to biomass,  
25 monosaccharides (e.g., glucose and fructose), disaccharides (e.g., lactose and sucrose),  
oligosaccharides, polysaccharides (e.g., starch and cellulose), as well as mixtures thereof,  
and unpurified mixtures from renewable feedstocks such as cheese whey permeate,  
cornsteep liquor, sugar beet molasses, and barley malt. In additional embodiments, the  
carbon substrate comprises one-carbon substrates such as carbon dioxide, or methanol for  
30 which metabolic conversion into key biochemical intermediates has been demonstrated.

Glycerol production from single carbon sources (e.g., methanol, formaldehyde or  
formate) has been reported in methylotrophic yeasts (See, Yamada *et al.*, Agric. Biol.  
Chem., 53:541-543 [1989]) and in bacteria (Hunter *et al.*, Biochem., 24:4148-4155 [1985]).  
These organisms can assimilate single carbon compounds, ranging in oxidation state from  
35 methane to formate, and produce glycerol. In some embodiments, the pathway of carbon

assimilation is through ribulose monophosphate, through serine, or through xylulose-monophosphate (See e.g., Gottschalk, Bacterial Metabolism, 2nd Ed., Springer-Verlag, New York [1986]). The ribulose monophosphate pathway involves the condensation of formate with ribulose-5-phosphate to form a 6-carbon sugar that becomes fructose and eventually  
5 the 3-carbon product glyceraldehyde-3-phosphate. Likewise, the serine pathway assimilates the one-carbon compound into the glycolytic pathway via methylenetetrahydrofolate.

In addition to the utilization of one and two carbon substrates, methylotrophic organisms are also known to utilize a number of other carbon-containing compounds such  
10 as methylamine, glucosamine and a variety of amino acids for metabolic activity. For example, methylotrophic yeast are known to utilize the carbon from methylamine to form trehalose or glycerol (Bellion *et al.*, in Murrell *et al.* (eds), 7<sup>th</sup> Microb. Growth C1 Compd., Int. Symp., 415-32, Intercept, Andover, UK [1993]). Similarly, various species of *Candida* metabolize alanine or oleic acid (Sulter *et al.*, Arch. Microbiol., 153:485-9 [1990]). Hence,  
15 the source of carbon utilized in the present invention encompasses a wide variety of carbon-containing substrates and is only limited by the requirements of the host organism.

Although it is contemplated that all of the above mentioned carbon substrates and mixtures thereof will find use in the methods of the present invention, preferred carbon  
20 substrates include monosaccharides, disaccharides, oligosaccharides, polysaccharides, and one-carbon substrates. In more particularly preferred embodiments, the carbon substrates are selected from the group consisting of glucose, fructose, sucrose and single carbon substrates such as methanol and carbon dioxide. In a most particularly preferred embodiment, the substrate is glucose.

As known in the art, in addition to an appropriate carbon source, fermentation media  
25 must contain suitable nitrogen source(s), minerals, salts, cofactors, buffers and other components suitable for the growth of the cultures and promotion of the enzymatic pathway necessary for the production of the desired end-product (e.g., glycerol). In some embodiments, (II) salts and/or vitamin B<sub>12</sub> or precursors thereof find use in the present invention.

30

### Culture Conditions

Typically, cells are grown at approximately 30 °C. in appropriate media. Preferred growth media utilized in the present invention include common commercially prepared media such as Luria Bertani (LB) broth, Sabouraud Dextrose (SD) broth or Yeast Malt

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Extract (YM) broth. However, other defined or synthetic growth media may also be used, as appropriate. Appropriate culture conditions are well-known to those in the art.

In some embodiments, agents known to modulate catabolite repression directly or indirectly (e.g., cyclic adenosine 2':3'-monophosphate or cyclic adenosine 2':5'-

5 monophosphate), are incorporated into the reaction media. Similarly, the use of agents known to modulate enzymatic activities (e.g., sulphites, bisulphites and alkalis) that lead to enhancement of glycerol production also find use in conjunction with or as an alternative to genetic manipulations.

Suitable pH ranges for fermentation are between pH 5.0 to pH 9.0; while the range of pH 6.0  
10 to pH 8.0 is particularly preferred for the initial conditions of the reaction system.

Furthermore, reactions may be performed under aerobic, microaerophilic, or anaerobic conditions, as suited for the organism utilized.

#### **Batch and Continuous Fermentations**

15 In some preferred embodiments, the present process uses a batch method of fermentation. A classical batch fermentation is a closed system, wherein the composition of the media is set at the beginning of the fermentation and is not subject to artificial alterations during the fermentation. Thus, at the beginning of the fermentation the medium is inoculated with the desired organism(s). In this method, fermentation is permitted to occur without the  
20 addition of any components to the system. Typically, a batch fermentation qualifies as a "batch" with respect to the addition of the carbon source and attempts are often made at controlling factors such as pH and oxygen concentration. The metabolite and biomass compositions of the batch system change constantly up to the time the fermentation is stopped. Within batch cultures, cells moderate through a static lag phase to a high growth  
25 log phase and finally to a stationary phase where growth rate is diminished or halted. If untreated, cells in the stationary phase eventually die. In general, cells in log phase are responsible for the bulk of production of end product or intermediate.

A variation on the standard batch system is the "fed-batch fermentation" system, which also finds use with the present invention. In this variation of a typical batch system,  
30 the substrate is added in increments as the fermentation progresses. Fed-batch systems are useful when catabolite repression is apt to inhibit the metabolism of the cells and where it is desirable to have limited amounts of substrate in the media. Measurement of the actual substrate concentration in fed-batch systems is difficult and is therefore estimated on the basis of the changes of measurable factors such as pH, dissolved oxygen and the partial

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pressure of waste gases such as CO<sub>2</sub>. Batch and fed-batch fermentations are common and well known in the art.

It is also contemplated that the methods of the present invention are adaptable to continuous fermentation methods. Continuous fermentation is an open system where a defined fermentation media is added continuously to a bioreactor and an equal amount of conditioned media is removed simultaneously for processing. Continuous fermentation generally maintains the cultures at a constant high density where cells are primarily in log phase growth.

Continuous fermentation allows for the modulation of one factor or any number of factors that affect cell growth and/or end product concentration. For example, in one embodiment, a limiting nutrient such as the carbon source or nitrogen level is maintained at a fixed rate and all other parameters are allowed to moderate. In other systems, a number of factors affecting growth can be altered continuously while the cell concentration, measured by media turbidity, is kept constant. Continuous systems strive to maintain steady state growth conditions. Thus, cell loss due to media being drawn off must be balanced against the cell growth rate in the fermentation. Methods of modulating nutrients and growth factors for continuous fermentation processes as well as techniques for maximizing the rate of product formation are well known in the art of industrial microbiology.

In some embodiments, the present invention is practiced using batch processes, while in other embodiments, fed-batch or continuous processes, as well as any other suitable mode of fermentation are used. Additionally, in some embodiments, cells are immobilized on a substrate as whole-cell catalysts and are subjected to fermentation conditions for the appropriate end-product production.

#### **Alterations in the Enzymatic Pathway**

Various alterations in enzymatic pathways are contemplated for use in the methods of the present invention. One representative enzyme pathway involves the production of 1,3-propanediol from glucose. In some embodiments, this is accomplished by the following series of steps which are representative of a number of pathways known to those skilled in the art. In this representative pathway, glucose is converted through a series of steps by enzymes of the glycolytic pathway to dihydroxyacetone phosphate (DHAP) and 3-phosphoglyceraldehyde (3-PG). Glycerol is then formed by either hydrolysis of DHAP to dihydroxyacetone (DHA) followed by reduction, or reduction of DHAP to glycerol 3-phosphate (G3P) followed by hydrolysis. The hydrolysis step can be catalyzed by any number of cellular phosphatases which are known to be specific or non-specific with respect

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to their substrates or the activity can be introduced into the host by recombination. In some embodiments, the reduction step is catalyzed by a NAD<sup>+</sup> (or NADP<sup>+</sup>)-linked host enzyme or the activity is introduced into the host by recombination. It is noted that the *dha* regulon contains a glycerol dehydrogenase (E.C. 1.1.1.6) which catalyzes the reversible reaction of

5 Equation 3.



10

Glycerol is converted to 1,3-propanediol via the intermediate 3-hydroxy-propionaldehyde (3-HP) as has been described in detail above. The intermediate 3-HP is produced from glycerol (Equation 1) by a dehydratase enzyme which can be encoded by the host or can be introduced into the host by recombination. This dehydratase can be glycerol dehydratase (E.C. 4.2.1.30), diol dehydratase (E.C. 4.2.1.28), or any other enzyme able to catalyze this transformation. Glycerol dehydratase, but not diol dehydratase, is encoded by the *dha* regulon. In some embodiments, 1,3-propanediol is produced from 3-HP (Equation 2) by a NAD<sup>+</sup> or NADP<sup>+</sup> linked host enzyme, while in other embodiments, the activity is introduced into the host by recombination. In some embodiments, this final reaction in the production of 1,3-propanediol is catalyzed by 1,3-propanediol dehydrogenase (E.C. 1.1.1.202) or other alcohol dehydrogenases. It is noted that in some embodiments, mutations and transformations affect carbon channeling. A variety of mutant organisms comprising variations in the 1,3-propanediol production pathway find use in the present invention. The introduction of a triosephosphate isomerase mutation (*tpi*-) into the microorganism is an example of the use of a mutation to improve the performance by carbon channeling. Alternatively, mutations which diminish the production of ethanol (*adh*) or lactate (*ldh*) increase the availability of NADH for the production of 1,3-propanediol.

30 Additional mutations in steps of glycolysis after glyceraldehyde-3-phosphate include the 1,3-propanediol production pathway. Mutations that effect glucose transport such as PTS which would prevent loss of PEP also find use in the present invention. Mutations which block alternate pathways for intermediates of the 1,3-propanediol production pathway such as the glycerol catabolic pathway (*glp*) also find use in the present invention. In some

35 embodiments, the mutation is directed toward a structural gene, so as to impair or improve

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the activity of an enzymatic activity or can be directed toward a regulatory gene so as to modulate the expression level of an enzymatic activity.

In additional embodiments, transformations and mutations are combined to as to control particular enzyme activities for the enhancement of 1,3-propanediol production.

- 5 Thus, it is within the scope of the present invention to provide modifications of a whole cell catalyst which lead to an increased production of 1,3-propanediol.

#### Identification and Purification of the End-Product

Methods for the purification of the end-product from fermentation media are known in  
10 the art. For example, propanediols can be obtained from cell media by subjecting the reaction mixture to extraction with an organic solvent, distillation and column chromatography (See e.g., U.S. Pat. No. 5,356,812). A particularly good organic solvent for this process is cyclohexane (See, U.S. Pat. No. 5,008,473).

In some embodiments, the end-product is identified directly by submitting the media  
15 to high pressure liquid chromatography (HPLC) analysis. One method of the present invention involves analysis of fermentation media on an analytical ion exchange column using a mobile phase of 0.01 N sulfuric acid in an isocratic fashion.

#### Identification and Purification of the Enzymes

20 The enzyme levels in the media can be measured by enzyme assays. For example in the manufacture of 1,3-propanediol, the levels of expression of the proteins G3PDH and G3P phosphatase are measured by enzyme assays. The G3PDH activity assay relies on the spectral properties of the cosubstrate, NADH, in the DHAP conversion to G-3-P. NADH has intrinsic UV/vis absorption and its consumption can be monitored spectrophotometrically  
25 at 340 nm. G3P phosphatase activity can be measured by any method of measuring the inorganic phosphate liberated in the reaction. The most commonly used detection method used the visible spectroscopic determination of a blue-colored phosphomolybdate ammonium complex.

Thus, although there are various superficial resemblances between the methods  
30 known in the art and the methods of the present invention, the present invention provides more comprehensive objectives that are reflected in a great number of detail features believed to be unique to practice of this invention, including notably enzyme recycling, biomass and starch recycling.

## Recovery

Overall, recovery of enzymes in recycled stillage requires care, in order to avoid  
subjecting the conversion media to temperatures that deactivate the enzymes. In one  
5 example, for the recovery of ethanol, the alcohol is vacuum stripped from the fermentation  
broth and the stillage is recycled, in order to recover the enzymes. In some embodiments,  
enzymes are recovered through the use of ultrafiltration or an electrodialysis device and  
recycled.

## 10 Process Considerations

As indicated above, fermentation of granular starch slurry has completely different  
characteristics than fermentation of a syrup. Generally, a concentration of about 20% solids  
in solution is considered the maximum sugar content in a fermentation medium, as higher  
concentrations create difficulties at the onset and at the end of fermentation. However, no  
15 similar limits exist in the fermentation of a starch slurry. The concentration of starch in the  
slurry may vary from 10 -35 %, with no discernable consequences at the onset of  
fermentation. Increasing starch concentration (*e.g.*, at constant enzyme dosages) speeds up  
the bioconversion rate, or conversely, allows for lowering the enzyme dosages required to  
achieve a given bioconversion rate. The excess (*i.e.*, residual) granular starch may be  
20 recovered, along with substantial amounts of enzymes and subjected to renewed  
fermentation. Thus, control over starch concentration is a major process parameter for  
practice of this invention.

In one preferred embodiment, means for bioconversion and fermentation of a  
granular starch slurry having 10-35% starch by weight are provided. In some preferred  
25 embodiments, fermentation of a 10-35% starch slurry with *E. coli* results in the production  
of residual starch when fermentation has proceeded to the intended organic acid or 1,3-  
propane diol content levels. However, this reaction is dependent on the microorganism and  
bioprocessing conditions used and, therefore, recycling of the enzymes on the starch  
particles occurs when the residual starch is again fermented. However, even when a 25-  
30 35% starch slurry is fermented, in preferred embodiments, the fermentation is halted before  
complete disappearance of the granular starch, for fermentation anew. Thus, recycling of  
starch is a facile way to recover enzymes for reuse.

In one preferred embodiment of the present invention, the (granular) starch and  
microorganisms are removed together (*e.g.*, by centrifugation or filtration). This removed

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starch and microorganisms are mixed with fresh granular starch and additional aliquot(s) of enzyme(s) as needed, to produce a fermentation charge for another fermentation run.

In another preferred embodiment, bioconversion and fermentation of a corn stover slurry having 10-25% cellulose by weight is provided. In one embodiment, fermenting a 10-25% cellulosic slurry with *E. coli* results in residual cellulose when fermentation has proceeded to the intended organic acid or 1,3-propane diol content levels. This reaction is dependent upon the microorganism and bioprocessing conditions used. As above, recycling of the enzymes on the cellulose occurs when the residual corn stover is again fermented. However, even when a 25-35% cellulose slurry is fermented, in some preferred  
5 10-25% cellulosic slurry with *E. coli* results in residual cellulose when fermentation has proceeded to the intended organic acid or 1,3-propane diol content levels. This reaction is dependent upon the microorganism and bioprocessing conditions used. As above, recycling of the enzymes on the cellulose occurs when the residual corn stover is again fermented. However, even when a 25-35% cellulose slurry is fermented, in some preferred  
10 embodiments, the fermentation is halted before the complete disappearance of the stover, for fermentation anew. Thus, recycling stover is a facile way to recover enzymes for reuse.

In yet another preferred embodiment, the corn stover and microorganisms are removed together (e.g., by centrifugation or filtration). This removed corn stover and microorganisms are mixed with fresh corn stover and additional aliquot(s) of enzyme(s) as  
15 needed, to produce a fermentation charge for another fermentation run.

As recognized by those of skill in the art, engineering trade-offs are contemplated in arriving at optimum process details; these trade-offs are contemplated to vary, depending upon each particular situation. Nonetheless, the methods provided herein provide the necessary teachings to make such trade-offs to obtain optimum processes. For example, to  
20 achieve the most rapid fermentation reasonable, high starch or cellulose content, and high enzymes dosage are indicated. But, the consequential rapid fermentation tails off into generation of a level of nutrients in the fermentation broth, when then dictates recovery of the nutrients, or, alternatively that fermentation be halted at a relatively low end-product (e.g., alcohol) content. However, in situations where relatively low fermentation rates are  
25 acceptable, then (with high starch content slurries) enzyme dosage is relatively low and nutrient losses are held to levels heretofore accepted by the fermentation arts. In cases where maximum yield of end-product (e.g., alcohol) is a principal objective, then low starch content slurries, moderate alpha-amylase dosage, and high glucoamylase dosage find use in the present invention. However, it is not intended that the present invention be limited to  
30 any particular method design.

As indicated herein, the present invention saves considerable thermal energy. However, just as the starting substrate (e.g., starch) is never subjected to the thermal conditions used for liquefactions, the substrate is not thermally sterilized. Thus, it is contemplated that in some embodiments, the starting substrate (e.g., granular starch) adds  
35 contaminating microorganisms to the fermentation medium. Thus, in some embodiments, it

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is advantageous to seed the fermentation medium with a large number of the product-producing microorganisms that are associated with recycled substrate (e.g., starch). By greatly outnumbering the contaminants, the recycled microorganisms overwhelm any contaminating microorganisms, thereby dominating the fermentation, resulting in the  
5 production of the desired end-product.

In some embodiments, the quantities of microorganisms and/or enzymes initially charged into the fermentation vat or bioreactor are in accord with prior art practices for the fermentation and/or bioconversion of various products. These quantities will vary, as the microbial cells multiply during the course of the fermentation whereas enzymes used for  
10 bioconversion will have a limited half-life. Although in some embodiments, recycling of microorganisms is utilized, in many embodiments, it is not required for the practice of the present invention. In contrast, in particularly preferred embodiments, it is desirable to recycle enzymes (although it is not intended that the present invention be limited to methods which require the recycling of enzymes).

Thus, in some embodiments, removal of the microbes from the residual starch or biomass particles prior to recycling of the residual starch or biomass is contemplated.  
However, it is again noted that practice of the present invention does not necessarily require thermal treatment of the starting substrate (e.g., starch). Thus, in some embodiments, the starting substrate is heat-sterilized, while in other embodiments, it is not. Therefore, in some  
20 embodiments, the fermentation/bioconversion is conducted in the presence of a relatively large proportion of microorganisms, in order to overcome the effects of any contamination. In alternative embodiments, antimicrobials are added to the fermentation medium to suppress growth of contaminating microorganisms. In additional embodiments, cold sterilization techniques, UV radiation, 65°C pasteurization are used to sterilize the starting  
25 (e.g., substrate) materials. However, biomass poses no problem regarding sterilization of fermentation vats or bioreactors.

As described herein, the present invention provides means to control the fermentation rate by releasing metabolizable sugars to the microbes or to subsequent enzymes at a controlled rate. The methods of the present invention are very different from  
30 what has been done heretofore. The prior art teaches the treatment of solid starch with enzymes prior to fermentation and/or inclusion of enzymes in the fermentation medium to conserve energy and/or to improve fermentation efficiency. However, in contrast to the present invention, there is no teaching in the art to alter the character of the fermentation so as to achieve a near to linear fermentation rate. The present invention provides means to  
35 efficiently conserve energy, particularly as compared to high temperature starch liquefaction.

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Indeed, in preferred embodiments, more thermal energy is conserved. The methods of the present invention operate with high fermentation efficiency, in part because product losses due to starch retrogradation, incomplete saccharification, and incomplete fermentation of fermentables are reduced. Furthermore, the ability to tailor the fermentation rate through  
5 control of starch or biomass concentration, as well as controlling the enzyme content and proportions, as provided by the present invention, facilitates the production of the desired end-products with minimal carbohydrate content.

As further indicated in the following Examples, the present invention provides novel methods for the production of gluconic acid using enzymatic conversion of starch. As  
10 indicated, using this enzymatic conversion of starch to gluconate helps remove two significant barriers currently encountered during the production of gluconate from glucose using enzymes. To compete with current gluconic acid production process, glucose needed to be used in 30-60 wt % solution, which partially inhibits glucose oxidase/catalase enzyme system at concentrations that high. In presently used methods, glucose concentrations this  
15 high result in a very high dosage of these enzymes and thus make the process economically prohibitive. An additional problem of currently used methods is that with use of 60% sugar solution substrates, there is a high viscosity level which negatively impacts solubility of oxygen in the reaction mixture. Oxygen is the second substrate and is required equimolarly for this oxidation. Lower availability of oxygen in the solution leads to lower rate  
20 of oxidation of glucose to gluconic acid and thus requires better  $K_{La}$  (oxygen delivering constant) delivering reactors.

Use of starch as the starting material does not only address the above shortcomings of currently used methods, but has at least three additional significant benefits in terms of the raw material cost of corn starch vs. D-glucose, reduction of substrate and/or product-  
25 based inhibition of enzymes employed in the bioconversion, and a concomitant significant reduction in the requirement of high enzyme dosage(s) for the production of gluconic acid.

In sum, the present invention provides novel methods for the production of gluconate from raw corn starch. Indeed, the present invention provides the first demonstration of the conversion of starch to gluconic acid using *in vitro* bioreactor and enzymatic bioconversion.  
30 The methods of the present invention further provide means for using a lower-cost renewable feed stock for the formation of a key commodity, namely industrial chemical gluconic acid.

In addition, the following Examples demonstrate that maltodextrin can be efficiently converted to gluconate using Genencor's OPTIMAX®, OXYGO® and FERMCOLASE®  
35 enzymes. It is also demonstrated that by using an optimized ratio of enzymes, the

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damaging effects of hydrogen peroxide produced during the reaction can be circumvented. In addition, the following Examples indicate that it is also possible to maintain the requisite dissolved oxygen requirement in the reactor for the oxidation of glucose produced from maltodextrin by configuring the enzyme dosages of all the three enzymes. It is also  
5 demonstrated that by optimizing the dosage of OPTIMAX (alpha amylase; Genencor), it is possible to control the release of glucose in the reaction mixture.

Furthermore, the following Examples demonstrate that fermentation control via alternate and cheaper carbon-feed stocks like starch, and biomass using enzyme-based conversion offers a more economical and efficient, as well as sustainable fermentation  
10 strategy to produce industrial chemicals, enzymes and therapeutics. As indicated in the following Examples, the rate of glucose release is controllable by the amount of enzyme addition. Indeed, it was observed that rate of starch conversion using glucoamylase was 100 fold faster than was initially predicted. However, the rate of glucose conversion to product is dependent upon the available glucose concentration in the medium and thus  
15 effects the final product formation. Thus, by controlling the release of glucose for available conversion by the amount of glucoamylase added, a means for manipulating the reaction to provide the fastest conversion rate achievable for product formation is provided.

In addition, the selectivity of conversion is controllable based on the dosage of glucoamylase used. As indicated in the following Examples, the best rate of product  
20 formation was produced using 3 units of enzymes. However, it is contemplated that the user of the present invention will modify the exact reaction conditions to suit their particular needs. Indeed, the details of each process are contemplated to vary, depending upon the kinetics of hydrolyzing enzymes used and the kinetics of glucose to product conversion. In addition, external reaction condition, such as pH, temp, and medium formulation are likewise  
25 important considerations. Nonetheless, the present invention provides the teachings necessary for the practice of the present invention under various conditions.

It is also contemplated that the methods of the present invention for efficient conversion of carbon feedstocks will find use in various other fermentations, including but not limited to the efficient production bioproducts from cellulose and/or hemicellulose. It is  
30 also contemplated that the starting materials provided herein will find use as substitutes for lactose in various fermentation processes. Thus, it is contemplated that the present invention will find wide-spread use in the industrial fermentation industry.

Various other examples and modifications of the description and Examples are apparent to a person skilled in the art after reading the disclosure without departing from the  
35 spirit and scope of the invention; it is intended that all such examples or modifications be

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included within the scope of the appended claims. All publications and patents referenced herein are hereby incorporated by reference in their entirety.

## EXPERIMENTAL

5 The following examples are provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof. Indeed, it is contemplated that these teachings will find use in further optimizing the process systems described herein.

In the experimental disclosure which follows, the following abbreviations apply: wt%  
 10 (weight percent); °C (degrees Centigrade); rpm (revolutions per minute); H<sub>2</sub>O (water); dH<sub>2</sub>O (deionized water); HCl (hydrochloric acid); aa (amino acid); bp (base pair); kb (kilobase pair); kD (kilodaltons); gm (grams); µg (micrograms); mg (milligrams); ng (nanograms); µl (microliters); ml and mL (milliliters); mm (millimeters); nm (nanometers); µm (micrometer); M (molar); mM (millimolar); µM (micromolar); U (units); V (volts); MW (molecular weight);  
 15 psi (pounds per square inch); sec (seconds); min(s) (minute/minutes); hr(s) (hour/hours); Q.S. and q.s. (quantity sufficient); OD (optical density); OD<sub>280</sub> (optical density at 280 nm); OD<sub>600</sub> (optical density at 600 nm); PAGE (polyacrylamide gel electrophoresis); DO (dissolved oxygen); Di (deionized); phthalate buffer (sodium phthalate in water, 20 mM, pH 5.0); PBS (phosphate buffered saline [150 mM NaCl, 10 mM sodium phosphate buffer, pH  
 20 7.2]); Cerestar granular starch (Cargill Foods PFP2200 granular starch; ); Cerestar (Cerestar, Inc., a Cargill Inc., company, Minneapolis, MN); AVICELL®(FMC Corporation); SDS (sodium dodecyl sulfate); Tris (tris(hydroxymethyl)aminomethane); w/v (weight to volume); v/v (volume to volume); slpm (standardized liters per minute); ATCC (American Type Culture Collection, Rockville, MD); Difco (Difco Laboratories, Detroit, MI); GIBCO BRL or Gibco BRL (Life Technologies, Inc., Gaithersburg, MD); Genencor (Genencor  
 25 International, Inc., Palo Alto, CA); Shin Nihon (Shin Nihon, Japan).

In the following examples, additional various media and buffers known to those in the art were used, including the following:

30 *Lactobacilli* MRS Media (for inoculum): Difco (Ref# 288130):  
0.5x Modified *Lactobacilli* MRS Media w/o glucose + 8% granular starch recipe:

Yeast extract (Difco)	15.0 g/L
Granular starch (Cerestar)	80.0 g/L
35 MgSO <sub>4</sub> *7H <sub>2</sub> O	0.3 g/L
KH <sub>2</sub> PO <sub>4</sub>	0.5 g/L
K <sub>2</sub> HPO <sub>4</sub>	0.5 g/L
Sodium acetate	0.5 g/L

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FeSO <sub>4</sub> *7H <sub>2</sub> O	0.03 g/L
MnSO <sub>4</sub> *1H <sub>2</sub> O	0.03 g/L
Mazu DF204 (antifoam)	1ml
1000x Tiger trace metal	0.2mls stock solution

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TM2 Recipe (per liter fermentation medium):

K<sub>2</sub>HPO<sub>4</sub> 13.6 g, KH<sub>2</sub>PO<sub>4</sub> 13.6 g, MgSO<sub>4</sub> \* 7H<sub>2</sub>O 2 g, citric acid monohydrate 2 g, ferric ammonium citrate .3 g, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 3.2 g, yeast extract 5 g, 1000X Modified Tiger Trace Metal Solution 1 ml. All of the components are added together and dissolved in diH<sub>2</sub>O. The pH is adjusted to 6.8 with potassium hydroxide (KOH) and q.s. to volume. The final product is filter sterilized with 0.22 u (micron) filter (only do not autoclave).

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Murphy III Medium (g/l)

KH<sub>2</sub>PO<sub>4</sub> (12g), K<sub>2</sub>HPO<sub>4</sub> (4 g), MgSO<sub>4</sub>.7H<sub>2</sub>O (2 g), DIFCO Soytone (2 g), sodium citrate (0.1 g), fructose (5 g), (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (1 g), nicotinic acid (0.02 g), 0.4 g/l FeCl<sub>3</sub>.6H<sub>2</sub>O (5 ml), and Pho salts (5 ml).

15

1000X Modified Tiger Trace Metal Solution:

Citric Acids \* H<sub>2</sub>O 40 g, MnSO<sub>4</sub> \* H<sub>2</sub>O 30 g, NaCl 10 g, FeSO<sub>4</sub> \* 7H<sub>2</sub>O 1 g, CoCl<sub>2</sub> \* 6H<sub>2</sub>O 1 g, ZnSO \* 7H<sub>2</sub>O 1 g, CuSO<sub>4</sub> \* 5H<sub>2</sub>O 100 mg, H<sub>3</sub>BO<sub>3</sub> 100 mg, NaMoO<sub>4</sub> \* 2H<sub>2</sub>O 100 mg. Each component is dissolved one at a time in Di H<sub>2</sub>O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilize with 0.22 micron filter.

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**EXAMPLE 1****Conversion of Glucose to Gluconate**

In this Example, experiments conducted to convert glucose to gluconate are describe. First, a 30 wt% glucose solution was produced (115 g of glucose in 275 ml of 50mM phthalate pH 5.12 in deionized H<sub>2</sub>O). This solution was held at 35°C and 0.3 bar of back-pressure. Then, 2700 U of glucose oxidase and 270 Units of catalase were mixed into the solution at 1100 rpm and 120 % DO (under normal temperature and pressure, NTP or ATP) dissolved oxygen in water ("DO"). Upon mixing the enzyme, the DO dipped below 15 % of saturation in the reaction medium under operating conditions indicating that with use of 30% glucose, oxygen can be a rate-limiting substrate. Indeed, it appeared that the that enzymes were partially inhibited when tested in solutions that were less than 30% sugar and picked up converting glucose as it went below 20% concentration. Thus, use of 60% sugar

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solution (*i.e.*, one of the most common sugar feeds utilized in the art) results in inhibition, as well as oxygen transfer challenges. The results of these experiments are shown in Figure 1.

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## EXAMPLE 2

### Conversion of Starch to Glucose

In this Example, experiments conducted to convert starch to glucose are described. First, a 30% corn starch slurry was made (100 grams of starch [Cerestar] were mixed in 270 ml of 50 mM phthalate buffer, pH 5.0), and was kept at 45°C. Then, the mixture was mixed at 1100 rpm and 150% DO. Then, 250 mg of RSH enzyme (CUCONC™; Japan; 187 glucoamylase Units/g of powder) were mixed into the solution. This combination resulted in an initial 16 g/l/hr conversion of starch to glucose at pH 5.0 and 45°C. These results indicate that RSH glucoamylase enzyme has excellent kinetics for starch to sugar conversion (See, Figure 2). However, it is contemplated that lower dosages of RSH glucoamylase will find use in the methods of the present invention to convert starch to glucose. Indeed, in some embodiments in which the 2 g/l/hr production commonly practiced in the art are used, 100 mg of RSH glucoamylase powder (activity/units) per liter of 30% starch stock solution is a sufficient amount to efficiently convert starch to glucose.

In additional experiments to assess the conversion of granular starch to glucose, an experiment was carried out in 1L orange cap bottle to monitor glucose formation from granular starch using enzymes with glucoamylase activity at desired fermentation pH 6.7 and temperature 34°C.

For this experiment, granular starch in slurry form, for maximum final concentration of 80 g/L glucose, was added to the 1L bottle (*e.g.*, a 300 mL slurry with 16% glucose equivalent starch was combined with 300 mL of TM2 medium; total of 48g Cerestar granular starch was added to the 600 ml slurry). The pH of the slurry/ broth was adjusted to 6.7 with NH<sub>4</sub>OH. The mixture was held at 34°C for 30 min for germination of any contaminant present in the starch slurry, and then pasteurized at 65°C for 14 hr. Then, the test enzymes (30ml UltraFilter concentrate of fermenter supernatant of a *Humicola grisea* run showing starch hydrolysis activity (*i.e.*, RSH activity) and 0.4ml of SPEZYME® FRED alpha amylase liquid concentrate (Genencor), as well as 30 mg spectinomycin and 1 mg vitamin B<sub>12</sub> (spectinomycin and B<sub>12</sub> were added as 0.2 micron filtered solution in DI water). During the reaction, samples were taken from the vessel, centrifuged, and the supernatants refrigerated to terminate the enzyme action. The supernatants were then subjected to HPLC analysis. This experiment monitored saccharification of granular starch by measuring

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glucose formation. The results indicated that 32.09g/L glucose accumulated in 3 hours. Thus, the conversion of granular starch to glucose at 10 g/L-hour rate was good for Simultaneous Saccharification and Fermentation (SSF) of granular starch to 1,3-propanediol at 34°C and pH 6.7.

5

### EXAMPLE 3

#### Conversion of Starch to Gluconate

In this Example, experiments conducted to convert starch to gluconate are described. First, a 30% corn starch slurry was made (100 gram of starch in 270 ml of 50 mM phthalate buffer, pH 5.1), and kept at 40°C. Then, under conditions of 1100 rpm and 130 DO, 250 mg of RSH enzyme (CUCONC™; Japan; 187 glucoamylase Units/g of powder), 880 ul of OXYGO® (glucose oxidase; Genencor) and 880 ul of FERMCOLASE® (catalase; Genencor) (1500 U/ml and 1000 U/ml) were mixed into the solution. This resulted in an initial 17 g/l/hr conversion of starch to glucose at pH 5.1-5.2 and 40°C. This result indicates that RSH glucoamylase enzyme has excellent kinetics for starch to sugar conversion under these bioconversion conditions in a bioreactor (See, Figure 3).

However, in additional embodiments, optimization of conditions helps maximize the long term stability of the system. Additional enzymes needed to convert glucose to gluconate were also determined to work well in unison with this system over the time course used in these experiments, as no glucose accumulation occurred. Thus, these results indicate that the dosage of the RSH enzyme required to run the process at volumetric productivity of 10 g/l/hr is much lower than is required in currently used methods.

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### EXAMPLE 4

#### Conversion of Starch to Gluconate with Added DISTILLASE®

In this Example, experiments conducted to convert starch to gluconate using DISTALLASE® in the enzyme mixture are described. First, a 30% corn starch (Cerestar) slurry was prepared in 10 mM acetate buffer (10 mM sodium acetate in water) pH 5.0, and brought to 40° C. Then, under conditions of 1100 rpm and 118 DO, 250 mg of CU CONC™ RSH glucoamylase, 150 ul of DISTILLASE®-L-400 (350 GAU/g; sp 1.15), 1250 ul of OXYGO®, and 1500ul of FERMCOLASE® were added to the solution. This resulted in an initial gluconate production rate of 25 g/l/hr. Thus, it is clear that addition of the DISTILLASE® L-400 glucoamylase enzyme to the reaction mixture helped improve not only

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the initial rate of gluconate production but also led overall improved conversion of raw corn starch to gluconic acid, as indicated in Figure 4.

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#### EXAMPLE 5

##### Conversion of Maltodextrin to Glucose

In order to further demonstrate the utility of the methods of the present invention, an alternate substrate was utilized. This substrate, maltodextrin, is also a key sugar source.

As shown in Figure 5, quantitative conversion of maltodextrin to glucose was feasible using  
10 OXYGO® and FERMCOLASE® enzymes.

#### EXAMPLE 6

##### Conversion of Maltodextrin to Gluconate

15 In addition, the conversion of maltodextrin to gluconate was attempted using low enzyme dosage conditions. In particular, a lower dose of catalase was tested. The results revealed that maltodextrin can be converted to gluconate in a single pot reaction using three enzymes (data not shown). In addition, it was determined that the OPTIMAX® (alpha amylase and pullulanase blend; Genencor) enzyme preparation is less sensitive to  
20 hydrogen peroxide, in comparison with CU CONC™ RSH glucoamylase tested in other Examples described herein.

#### EXAMPLE 7

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##### Ratio of OXYGO® and FERMCOLASE® Enzymes

In further experiments, it was determined that a minimal 1:1 ratio of activity basis is desired for maximal productivity and stability of OXYGO® enzyme. As indicated in Figure 6, complete conversion of glucose to gluconate was demonstrated under these conditions.

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#### EXAMPLE 8

##### Maltodextrin to Gluconate Conversion Using Reestablished Enzyme Dosage

In this experiment, production of gluconate from maltodextrin was achieved to a yield  
35 of > 50%, at a rate of 7 g/l/hr. Initial conversion rates approached to more than 25 g/l/hr.

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The dosage level used in this example was 1000 Units of OXYGO® enzyme and FERMCOLASE® enzyme with 200 Units of OPTIMAX® enzyme. This example illustrates the need to utilize the correct enzyme types to achieve the bioconversion.

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#### EXAMPLE 9

##### Optimization of Enzyme Dosages to Improve the Overall Conversion Efficiency

In these experiments, the production yield and volumetric productivity of gluconate from maltodextrin reached to over 80% and 8 g/l/hr by further optimizing the dosage of OPTIMAX® enzyme (See, Figure 8). The dosage level used in this example was 1250 Units of OXYGO® and 1000 Units FERMCOLASE® with 200 Units of OPTIMAX®. This example illustrates the need to include the correct enzyme type(s) and the dosage level optimization to achieve desired bioconversion.

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#### EXAMPLE 10

##### Comparison Between Raw Corn Starch and Raw Wheat Starch

In order to further demonstrate the utility of the methods of the present invention, an alternate starch source was examined. This substrate, raw wheat starch, is also a key sugar source. As shown in Figure 9, wheat starch can also be efficiently converted to gluconate using OXYGO®, FERMCOLASE®, DISTILLASE®, and CU CONC RSH glucoamylase enzymes. Indeed, the results indicate that wheat starch is more amenable to bioconversion than corn starch when compared for the similar bioconversion time.

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#### EXAMPLE 11

##### Conversion of Starch to Lactic Acid

This experiment was carried out in 1L bioreactor to monitor lactate formation from granular starch using enzymes with glucoamylase activity at desired fermentation pH 6.4 and temperature 34°C. In this experiment, granular starch in slurry form (maximum final concentration of 80 g/L glucose) in the 0.5x modified *Lactobacilli* medium fermentation medium, was pasteurized (*i.e.*, the mixture was held at 34°C for 30 min for germination of any contaminant present in the starch slurry, and then pasteurized at 65 °C for 14 hr). This was added to the pre-sterilized 1L bioreactor. The pH of the slurry/broth was adjusted to 6.4

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and controlled at 6.4 with 28% NH<sub>4</sub>OH. Then, the desired enzymes (0.4g of sumizyme CU CONC™; Shin Nihon) were added as 0.2 micron filtered solution (20ml) in DI water. Then, an inoculum of lactate-producing strain *Lactobacillus casei* (ATCC 393), taken from a frozen vial, was prepared in *Lactobacillus* MRS medium (Difco). After the inoculum grew to OD 2.4, measured at 550 nm, in a 1L bioreactor at 34°C with a nitrogen sparge at 0.6 slpm (standardized liters per minute) flow rate), the contents of the reactor (600ml) were centrifuged and re-suspended in 45ml supernatant to transfer the cell pellet (42ml of OD22 material) as the inoculum for the fermentative bioconversion in a bioreactor. For the duration of the fermentative bioconversion run, nitrogen was sparged at 0.6 slpm, the back pressure was held at 5psi, the temperature was held at 34°C, pH held at 6.4 by base titration of 28% NH<sub>4</sub>OH.

During the reaction, samples were taken from the vessel, centrifuged, and the supernatants were refrigerated to terminate the enzyme action. The supernatant was then subjected to HPLC analysis. This experiment monitored bioconversion of granular starch by measuring glucose formation and its conversion to lactate. In 16.3 hours, accumulation of lactate amounted to 61.75 g/L (Figure 10).

In addition, the bioconversion of granular starch to lactate was demonstrated to be at a level of 3.79 g/L-hour rate, at a temperature of 34 °C, and at pH 6.4.

## EXAMPLE 12

### Conversion of Starch to Succinic Acid

This experiment was carried out in 1L bioreactor to monitor succinate formation from granular starch using enzymes with glucoamylase activity at desired fermentation conditions of pH 6.7 and temperature 34 °C.

For this experiment, raw granular starch in slurry form (maximum final concentration 80 g/L glucose) in 0.5x TM2 fermentation medium, was pasteurized (*i.e.* the mixture was held at 34 °C for 30 min for germination of any contaminant present in the starch slurry, and then pasteurized at 65 °C for 14 hr). This was added to the pre-sterilized 1L bioreactor. The pH of the slurry/ broth was adjusted to 6.7 and controlled at 6.65 with NH<sub>4</sub>OH. Then, the desired enzymes (0.6g of sumizyme CU CONC; Shin Nihon) were added as 0.2 micron filtered solution (20ml) in DI water. An inoculum of succinate -producing strain 36 1.6ppc *E. coli*, taken from frozen vial, was prepared in TM2 + 10g/L glucose medium. After the inoculum grew to OD 0.6, measured at 550 nm, one 600ml flask was centrifuged and re-suspended in 80ml supernatant to transfer the cell pellet (80ml of OD 14.3 material) to the

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bioreactor. At 3.7 hours in to the run, the air being sparged at 0.6 slpm was switched to nitrogen, which was also sparged at 0.6 slpm.

During the reaction, samples were taken from the vessel, centrifuged and the supernatants were refrigerated to terminate the enzyme action. The supernatant were  
5 subjected to HPLC analysis. This experiment monitored bioconversion of granular starch by measuring glucose formation and its conversion to succinate. In 43 hours, accumulation of succinate amounted to 1.46 g/L (Figure 11). The conversion of granular starch to succinate at 0.034 g/L-hour rate was demonstrated for fermentative bioconversion of granular starch to succinate at 34 °C and pH 6.7.

10

### EXAMPLE 13

#### Conversion of Starch to 1,3-Propanediol

This experiment was carried out in 1L bioreactor to monitor 1,3-propanediol  
15 formation from granular starch using enzymes with glucoamylase activity at the desired fermentation pH 6.7 and temperature 34 °C.

For this experiment, granular starch in slurry form (for maximum final concentration 80 g/L glucose) in 0.5x TM2 fermentation medium, was pasteurized as described above (i.e., the mixture was held at 34 °C for 30 min for germination of any contaminant present in  
20 the starch slurry, and then pasteurized at 65 °C for 14 hr). This was added to the pre-sterilized 1L bioreactor. The pH of the slurry/broth was adjusted to 6.7 and controlled at 6.65 with NH<sub>4</sub>OH. Then, the desired enzymes (30ml UltraFilter concentrate of fermenter supernatant of a *Humicola grisea* run with starch hydrolysis activity [i.e., RSH glucoamylase activity] and 0.4ml of SPEZYME® FRED liquid concentrate [Genencor] having alpha  
25 amylase activity), and requirements specific for 1,3-propanediol production (30 mg spectinomycin and 2 mg vitamin B<sub>12</sub>) were added as 0.2 micron filtered solution in DI water. An inoculum of 1,3-propanediol-producing *E. coli* strain TTaldABml/p109F1 taken from a frozen vial, was prepared in soytone-yeast extract-glucose medium. After the inoculum grew to OD 0.6, measured at 550 nm, two 600ml flasks were centrifuged and the contents  
30 resuspended in 70ml supernatant to transfer the cell pellet (70ml of OD3.1 material) to the bioreactor.

During the reaction, samples were taken from the vessel, centrifuged, and supernatants refrigerated to terminate the enzyme action. The supernatants were then subjected to HPLC analysis. This experiment monitored fermentative bioconversion of  
35 granular starch by measuring glucose formation and its conversion to glycerol (1,3-

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propanediol pathway intermediate) and then to 1,3-propanediol. In 23.5 hours, accumulation of glycerol and 1,3-propanediol amounted to 7.27 and 41.93 g/L, respectively (Figure 12).

Conversion of granular starch to glycerol and 1,3-propanediol at 1.75 g/L-hour rate  
5 was demonstrated for fermentative bioconversion of granular starch to 1,3-propanediol at 34 °C and pH 6.7.

In additional similar experiments, the fermentative bioconversion of granular starch to glycerol was determined at 34° and pH 6.7. In these experiments, glucose formation and its conversion to glycerol were determined. In nine hours, the accumulation of glycerol was  
10 found to be 14.93 g/L. The conversion rate of granular starch to glycerol was 1.60 g/L-hour, a good rate for fermentative bioconversion of granular starch. Likewise, the 1.75 g/L-hour rate indicated above, was found to be a good rate for fermentative bioconversion of granular starch to 1,3-propanediol.

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#### EXAMPLE 14

##### **Fermentative Bioconversion of Starch to 1,3-Propanediol By CU CONC RSH Glucoamylase**

The first experiment was carried out in 1L orange cap bottles to monitor glucose  
20 formation from granular starch using enzymes with glucoamylase activity at desired fermentation pH 6.7 and temperature 34°C.

For this experiment, granular starch in slurry form (for maximum final concentration 40 g/L glucose), was added to the 1L bottle (e.g., 300 mL slurry with 8% glucose equivalent starch) and combined with 300 mL of TM2 medium. The pH of the slurry/broth was adjusted  
25 to 6.7 with NH<sub>4</sub>OH. The mixture was held at 34-35°C for 30 min for germination of any contaminants present in the starch slurry, and then pasteurized at 65°C for 14 hr. Then, the desired enzymes (0.6g Sumizyme CU; Shin Nihon), and requirements specific for 1,3-propanediol production (30 mg spectinomycin and 1 mg vitamin B<sub>12</sub>) were added as 0.2 micron filtered solution in DI water. During the reaction, samples were taken from the  
30 vessel, centrifuged, and the supernatants refrigerated to terminate the enzyme action. The supernatants were then subjected to HPLC analysis. This experiment monitored bioconversion of granular starch by measuring glucose formation. In this experiment, 12.86 g/L glucose accumulated in 6 hours. Conversion of granular starch to glucose at 2 g/L-hour rate was demonstrated for bioconversion of granular starch to 1,3-propanediol at 35°C and  
35 pH 6.7 (data not shown).

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In a second experiment, a 1L bioreactor was used to monitor 1,3-propanediol formation from granular starch using enzymes with RSH glucoamylase activity at a desired fermentation pH 6.7 and temperature 34 °C. For this experiment, granular starch in slurry form (for maximum final concentration 40 g/L glucose) in TM2 fermentation medium, was  
5 sterilized and pasteurized as described above. This mixture was added to the pre-sterilized 1L bioreactor. The pH of the slurry/broth was adjusted to 6.7 and controlled at 6.65 with NH<sub>4</sub>OH. The mixture was held at 34°C for 30 min for germination of any contaminants present in the starch slurry, and then pasteurized at 65°C for 14 hr. Then, the desired enzyme (0.6g Sumizyme CU; Shin Nihon), and requirements specific for 1,3-propanediol  
10 production (30 mg spectinomycin and 1 mg vitamin B12) were added as 0.2 micron filtered solution in DI water. An inoculum of 1,3-propanediol-producing *E. coli* strain FMP'ml(1.5gap)/pSYCO106 taken from a frozen vial, was prepared in soytone-yeast extract-glucose medium. After the inoculum grew to OD 1.1, measured at 550 nm, cells were centrifuged to transfer the cell pellet to the bioreactor.

15 During the reaction, samples were taken from the vessel, centrifuged, and the supernatants refrigerated to terminate the enzyme action. The supernatants were then subjected to HPLC analysis. This experiment monitored fermentative bioconversion of granular starch by measuring glucose formation and its conversion to glycerol (1,3-propanediol pathway intermediate) and then to 1,3-propanediol. In 5 hours, the  
20 accumulation of glycerol and 1,3-propanediol amounted to 2.57 and 0.59 g/L, respectively (Figure 13).

These results indicated good conversion of granular starch to glycerol and 1,3-propanediol at a 0.63 g/L-hour rate for fermentative bioconversion of granular starch to 1,3-propanediol at 34 °C and pH 6.7.

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## EXAMPLE 15

### Fermentative Bioconversion of Starch to Glycerol

This experiment was carried out in a 1L bioreactor to monitor 1,3-propanediol  
30 formation from granular starch using enzymes with glucoamylase activity at desired fermentation pH 6.7 and temperature 34 °C.

For this experiment, granular starch (Cerestar) in slurry form (for maximum final concentration 80 g/L glucose) in 0.5x TM2 fermentation medium, was pasteurized as described above (*i.e.*, the mixture was held at 34°C for 30 min for germination of any  
35 contaminants present in the starch slurry, and then pasteurized at 65°C for 14 hr). This

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mixture was then added to the pre-sterilized 1L bioreactor. The pH of the slurry/broth was adjusted to 6.7 and controlled at 6.65 with NH<sub>4</sub>OH. Then, the desired enzymes (30ml UltraFilter concentrate of a fermenter supernatant obtained from a culture of *Humicola grisea* showing starch hydrolysis activity [*i.e.*, RSH activity] and also 0.4ml of SPEZYME®  
5 FRED liquid concentrate [Genencor] having alpha amylase activity), and 30 mg spectinomycin were added as 0.2 micron filtered solution in DI water. An inoculum of glycerol producing *E. coli* strain TTaldABml/p109F1, was prepared in soytone-yeast extract-glucose medium (Difco). After the inoculum grew to OD 0.6, measured at 550 nm, two 600ml flasks were centrifuged and resuspended in 70ml supernatant to transfer the cell  
10 pellet (70ml of OD3.1 material) to the bioreactor.

During the reaction, samples were taken from the vessel, centrifuged, and the supernatants refrigerated to terminate the enzyme action. The supernatants were then subjected to HPLC analysis. This experiment monitored fermentative bioconversion of granular starch by measuring glucose formation and its conversion to glycerol. In 9 hours,  
15 the accumulation of glycerol amounted to 14.93 g/L (Figure 14). The conversion of granular starch to glycerol at 1.60 g/L-hour rate was demonstrated for fermentative bioconversion of granular starch to glycerol at 34°C and pH 6.7.

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## EXAMPLE 16

### Conversion of Starch to 2,5-DKG

In this Example, a fermentative bioprocess using corn starch and a RSH glucoamylase is demonstrated to maintain a rate of glucose release which will suffice the maximum production rate of a product such as 2,5-diketo-D-gluconic acid, a precursor  
25 molecule of vitamin C, using a microorganism known as *Pantoea citrea*.

Cerestar raw corn starch and M1Biocon (India) glucoamylase (1786 Gau/g) were used in this study. *Pantoea citrea* (a Gram-negative bacterial species with periplasmic oxidative dehydrogenases needed for producing oxidative sugar keto acid products such as 2,5-Diketo L-gluconic acid (2,5-DKG) and 2-keto L-gluconic acid 2-KLG from glucose) was  
30 used in this Example.

Murphy-III medium was used to grow the cells overnight. A modified Murphy-III medium (see below for formula) was used for the starch to glucose to 2,5-DKG conversion. Shake-flasks and rotary shakers were used in these experiments. Product analyses were performed using HPLC (Water's), and glucose was analyzed enzymatically using the  
35 Monarch robotics system (*i.e.*, an instrument known in the art for automated assay work).

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*Pantoea citrea* was inoculated in 100 ml of Murphy-III medium [at 28°C and 250 rpm overnight. Five-flasks containing 40 ml of deionized water (DI) and 1 gram of raw corn starch (20g/l final concentration) were pasteurized as described above (i.e. the mixture was held at 34 °C for 30 min for germination of any contaminant present in the starch slurry, and then pasteurized at 65 °C for 14 hr).

Modified Murphy-III medium was used to provide medium for both further growth of cells and product formation was prepared. Filter-sterilized 10x medium consisted of (per liter), KH<sub>2</sub>PO<sub>4</sub>, 24 g; K<sub>2</sub>HPO<sub>4</sub>, 8g; MgSO<sub>4</sub>, 0.16g; MSG, 1.5 g; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1g; nicotinic acid; Pho salts (CaCl<sub>2</sub>, MnCl<sub>2</sub>, NaCl); FeCl<sub>3</sub>; pantothenate and tetracycline 20mg/l. The pH of the medium was adjusted to 5.8 using potassium phosphate. Then, 5 ml of this medium were aseptically added to the shake-flasks containing the starch and water mixture. In another flask, 40ml of water containing 1 gram of glucose and 5 ml of the modified Murphy-III medium were added aseptically. Then, 5 ml of cell culture which grew to an OD of 21.5 at 550 nm overnight were then added to five-flasks. Flask-1 (GCMK1) thus contained 20g/l glucose and 5ml of *P. citrea* cell culture in modified Murphy-III medium. Flask-2 (GCMK2) contained 1 g of starch, 5ml of cell culture and the reaction was started with addition of 10 units of Biocon glucoamylase. Flask-3 (GCMK3) was the same as flask-2 except it also contained 3 units of glucoamylase. Flask-4 (GCMK4) had an added 1 unit of glucoamylase. Flask-5 (GCMK5) was a control, with no glucoamylase added. Flask-6 (GCMK6) was another control, in which 1 unit of glucoamylase was added but no cells were added. At three time points (0.3 hrs, 3hrs, and 7hrs) during incubation, 1.5 ml samples were withdrawn from each flask and were centrifuged. The supernatants were then filtered and processed for product analysis, pH, and glucose measurements. The results are shown in Figure 15.

The results indicated that corn starch is a suitable carbon source in fermentation control and production of 2,5-DKG using *P. citrea* cells and glucoamylase. Flasks 4 and 6, which contained 1 unit of glucoamylase had similar glucose levels of 5.6 g/l. This glucose level translates to a 20 g/l/hr conversion rate. Thus, Flask-2 with 10 units of glucoamylase had 15 g/l of glucose within 0.3 hr. The results of Flask-1 (with added glucose) were similar to those obtained with Flask-2. The rate of glucose production in Flask-3 correlated well with Flasks 2 and 4. As expected, Flask-5 had no glucose.

At three-hour time point, glucose levels in Flasks 1-4 dropped below 1 g/l and were converted to oxidative products gluconic acid, 2KDG and 2,5-DKG. It was interesting to note that Flask-2, 3 and 4, with controlled release of glucose, demonstrated greater end-product formation whereas Flask-1 with excess glucose produced lower levels of end

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product formation, but still had higher product intermediate concentrations. Control Flasks 5, and 6 behaved as expected.

By the seven hour sampling time point, each of Flasks 1-4 produced the expected product levels. In addition, the pH dropped in Flasks 1-4 and the trends were as expected  
5 based on the product (sugar acid) formation

### EXAMPLE 17

#### Bioconversion of Cellulosic Biomass to Gluconic Acid

10 As indicated in this Example, cellulose derived from biomass such as AVICEL® (FMC Corporation) and corn stover can be converted to a desired end-product using biocatalytic systems. This method for converting biomass overcomes product inhibition of cellulolytic enzymes during the conversion of biomass to glucose. This process converts the cellulolytic end-products concomitantly to the desired final product so that inhibition of  
15 cellulolytic enzymes is minimized. Cellulosic end-products such as glucose, xylose and cellobiose are produced, but are converted at the same time and rate to the final product, thereby allowing minimal accumulation of these end products which are also inhibitory to cellulolytic enzymes. Thus, the present method provides improved productivity and yield of the desired end-product

20 In these experiments, cellulose (AVICEL®; 30 g 10 wt%) and corn stover ( 30 g, 10 wt%) were tested in separate experiments, in 270 g of 50 mM citrate buffer pH 5.0 in a 1 liter bioreactor at 45° C equipped with pH, stirring, temperature, foam and oxygen control. Conversion of cellulose to glucose was started by adding 10 ml (dosed at 30 mgs of total protein per gram of cellulose) of SPEZYME® CP (Genencor) and the degree of hydrolysis  
25 was measured over the course of the reaction. In a subsequent experiment, 1.5 ml OXYGO® glucose oxidase (Genencor) and 2 ml FERMCOLASE® catalase (Genencor) were mixed along with 10 ml SPEZYME® CP (Genencor) were added to the cellulose and corn stover. These enzymes were found to convert the cellulose and corn stover to gluconic acid at an improved rate, as compared to the rate of glucose production from cellulose in a  
30 control experiment. This allowed the steady-state concentration of glucose in the reaction to remain at an essentially non-existent level. The gluconic acid concentration was measured using HPLC and the degree of hydrolysis was back calculated. The results established that in the same period of time where 30 g/l glucose was made from AVICEL®, in the control experiment (See, Figures 16A and 16B), over 50 g/l gluconic acid from AVICEL® was made  
35 using enzyme blend of OXYGO®, FERMCOLASE®, and SPEZYME®. In a 48 hr time

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frame, 60 wt% tech grade AVICEL® (Lattice 20) was converted to gluconic acid (Figure 16B). It was observed that by keeping the cellulosic end-product concentration at a minimum, it is possible to keep the cellulose hydrolyzing enzymes stable during the time course of the reaction.

5

### EXAMPLE 18

#### Fermentative Bioconversion of Biomass to 1, 3-Propanediol

This Example experiments to determine the suitability of using bioconversion to produce 1,3-propane diol from biomass are described. These experiments were carried out in a 2L tri-baffled Erlenmeyer flask to monitor glucose formation from cellulose (technical grade, AVICEL® Lattice 20) using enzymes with cellulase activity at desired fermentation pH 6.7, at 34 °C.

For this experiment, cellulose in slurry form (for maximum final concentration 100 g/L glucose), was added to the 2L flask (e.g., 200 mL slurry with 20% cellulose) was combined with 200 mL of TM2 medium (to give a 100 g/L glucose equivalent). The pH of the slurry/broth was adjusted to 6.7 with NH<sub>4</sub>OH. The mixture was sterilized at 121°C for 30 min. Then, the desired enzyme (13ml SPEZYME® CP; Genencor), and requirements specific for 1,3-propanediol production (20 mg spectinomycin and 1 mg vitamin B12) were added as a 0.2 micron filtered solution in DI water. During the reaction, samples were taken from the vessel, centrifuged, and the supernatants refrigerated to terminate enzyme action. The supernatants were subjected to HPLC analysis. This experiment monitored degradation of biomass (cellulose) by measuring glucose formation. It was determined that 12.19 g/L glucose accumulated in 98.7 hours. Conversion of biomass to glucose at a 0.12 g/L-hour rate was demonstrated for bioconversion of biomass to 1,3-propanediol at 34°C and pH 6.7 (data not shown).

Subsequently, an experiment was carried out in a 1L bioreactor to monitor glucose formation from cellulose (technical grade, AVICEL®) using enzymes with cellulase activity at desired fermentation pH 6.7 and temperature 34 °C. In this experiment, biomass (cellulose) in slurry form (for maximum final concentration 100 g/L glucose) in TM2 fermentation medium, was sterilized in the 1L bioreactor. The pH of the slurry/broth was adjusted to 6.7 and controlled at 6.65 with NH<sub>4</sub>OH. The mixture was sterilized at 121°C for 30 mins. Then, the desired enzymes (22ml SPEZYME® CP; Genencor), and requirements specific for 1,3-propanediol production (30 mg spectinomycin and 1 mg vitamin B12) were added as 0.2 micron filtered solution in DI water. An inoculum of 1,3-propanediol-producing *E. coli* strain

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TTaldABml/p109f1 WS#2 taken from a frozen vial, was prepared in soytone-yeast extract-glucose medium (Difco). After the inoculum grew to OD 1.2, measured at 550 nm, 60mls of broth were transferred to the bioreactor.

During the reaction, samples were taken from the vessel, centrifuged, and the supernatants refrigerated to terminate the enzyme action. The supernatants were subjected to HPLC analysis. This experiment monitored fermentative bioconversion of biomass to 1,3-propanediol by measuring glucose formation and its conversion to glycerol (1,3-propanediol pathway intermediate) and then to 1,3-propanediol. In 24.4 hours, the accumulation of glycerol and 1,3-propanediol amounted to 1.02 and 4.73 g/L, respectively (See, Figure 17).

The conversion of biomass to glycerol and 1,3-propanediol at 0.24 g/L-hour rate was demonstrated for fermentative bioconversion of biomass to 1,3-propanediol at 34°C and pH 6.7.

#### EXAMPLE 19

##### Fermentative Bioconversion of Biomass to Lactic Acid

This experiment was carried out in a 1L bioreactor to monitor glucose formation from cellulose (technical grade, AVICEL® Lattice 20) using an enzyme with cellulase activity at desired fermentation pH 6.4 and temperature 34°C, and the subsequent conversion to lactate using the lactate producing strain *Lactobacillus casei*.

For this experiment, biomass (cellulose) in slurry form (for maximum final concentration 100 g/L glucose) in the modified *Lactobacilli* MRS medium, was sterilized in the 1L bioreactor. The pH of the slurry/broth was adjusted to 6.4 and controlled at 6.4 with 28% NH<sub>4</sub>OH. The mixture was sterilized at 121°C for 30min. After cooling to a run temp of 34°C, the desired enzyme (22ml SPEZYME® CP; Genencor) was added as 0.2 micron filtered solution in DI water. An inoculum of lactate producing strain *Lactobacillus casei* (ATCC 393), taken from a frozen vial, was prepared in *Lactobacilli* MRS medium (Difco). After the inoculum grew to OD 2.7, measured at 550 nm, in a 1L bioreactor at 34°C with a nitrogen sparge at 0.6 slpm, the contents of the reactor (600ml) were centrifuged and re-suspended in 50ml supernatant to transfer the cell pellet (46ml of OD 24.2 material) as the inoculum for the SDC bioreactor.

During the reaction, samples were taken from the vessel, centrifuged, and the supernatants were refrigerated to terminate the enzyme action. The supernatants were subjected to HPLC analysis. This experiment monitored fermentative bioconversion of

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biomass to lactate by measuring glucose formation and its conversion to lactate. In 48 hours, accumulation of lactate amounted to 3.93 g/L (Figure 18).

5

**EXAMPLE 20****Fermentative Bioconversion of Biomass to Succinic Acid**

This experiment was carried out in a 1L bioreactor to monitor glucose formation from cellulose (technical grade, AVICEL® Lattice 20) using enzymes with cellulase activity at desired fermentation pH 6.7 and temperature 34°C, and the subsequent conversion to succinate, using the succinate producing strain, 36 1.6 ppc (*E. coli*).

For this experiment, biomass (cellulose) in slurry form (for maximum final concentration 100 g/L glucose) in the TM2 fermentation medium, was sterilized in the 1L bioreactor. The pH of the slurry/ broth was adjusted to 6.7 and controlled at 6.65 with NH<sub>4</sub>OH. The mixture was sterilized at 121°C for 30min. After cooling to a run temp of 34°C, the desired enzyme (22ml SPEZYME® CP; Genencor) were added as 0.2 micron filtered solution in DI water. An inoculum of succinate-producing strain 36 1.6ppc *E. coli*, taken from a frozen vial, was prepared in TM2 + 10g/L glucose medium. After the inoculum grew to OD 0.85, measured at 550 nm, the contents of one 600ml flask was centrifuged and re-suspended in 60ml supernatant to transfer the cell pellet (60ml of OD 9.3 material) to the bioreactor. For the duration of the run, nitrogen was sparged at 0.6 slpm

During the reaction, samples were taken from the vessel, centrifuged and supernatants were refrigerated to terminate the enzyme action. The supernatant was subjected to HPLC analysis. This experiment monitored fermentative bioconversion of biomass to succinate by measuring glucose formation and its conversion to succinate (See, Figure 19). In 48 hours, accumulation of succinate amounted to 2.73 g/L.

**CLAIMS**

1. A method for producing an end-product comprising the steps of:
  - a) contacting a carbon substrate and at least one substrate-converting enzyme to produce an intermediate; and
  - b) contacting said intermediate with at least one intermediate-converting enzyme, wherein said intermediate is substantially all converted by said intermediate enzyme to said end-product.
2. The method of Claim 1, wherein said intermediate-converting enzyme is a microbial enzyme.
3. The method of Claim 2, wherein said intermediate-converting microbial enzyme is secreted by a microorganism in contact with said intermediate.
4. The method of Claim 1, wherein said substrate-converting enzyme is a microbial enzyme.
5. The method of Claim 4, wherein said substrate-converting microbial enzyme is secreted by a microorganism in contact with said substrate.
6. The method of Claim 1, wherein said intermediate-converting enzyme and said substrate-converting enzyme are produced by microorganisms of the same species.
7. The method of Claim 1, wherein said intermediate-converting enzyme and said substrate-converting enzyme are produced by microorganisms of the different species.
8. The method of Claim 1, wherein concentration level of said intermediate is maintained at a level below that which triggers catabolite repression effects upon the conversion of said intermediate to said end-product.
9. The method of Claim 1, wherein concentration level of said intermediate is maintained at a level below that which triggers enzymatic inhibition effects upon the conversion of said intermediate to said end-product.

10. The method of Claim 1, wherein said intermediate is converted to said end-product at a rate sufficient to maintain the concentration of said at less than 0.25%.
11. The method of Claim 1, wherein said substrate is selected from the group consisting of biomass and starch.
12. The method of Claim 1, wherein said intermediate is selected from the group consisting of hexoses and pentoses.
13. The method of Claim 12, wherein said hexose is glucose.
14. The method of Claim 1, wherein said end-product is selected from the group consisting of 1,3-propanediol, gluconic acid, glycerol, succinic acid, lactic acid, 2,5-diketo-D-gluconic acid, gluconate, glucose, alcohol, and ascorbic acid intermediates.
15. The method of Claim 1, wherein said contacting said substrate and substrate-converting enzyme further comprises bioconverting said substrate to produce said intermediate.
16. A method for producing an end-product comprising the steps of:
  - a) contacting a carbon substrate and at least one substrate-converting enzyme to produce an intermediate; and
  - b) contacting said intermediate with at least one intermediate-converting enzyme, wherein said intermediate is substantially all converted by said intermediate enzyme to said end-product, and wherein the presence of said end-product does not inhibit the further production of said end-product.
17. The method of Claim 16, wherein said intermediate-converting enzyme is a microbial enzyme.
18. The method of Claim 16, wherein said intermediate-converting microbial enzyme is secreted by a microorganism in contact with said intermediate.
19. The method of Claim 16, wherein said substrate-converting enzyme is a microbial enzyme.

20. The method of Claim 16, wherein said substrate-converting microbial enzyme is secreted by a microorganism in contact with said substrate.

21. The method of Claim 16, wherein said intermediate-converting enzyme and said substrate-converting enzyme are produced by microorganisms of the same species.

22. The method of Claim 16, wherein said intermediate-converting enzyme and said substrate-converting enzyme are produced by microorganisms of the different species.

23. A method for producing an end-product comprising the steps of:

- a) contacting a carbon substrate and at least one substrate-converting enzyme to produce an intermediate; and
- b) contacting said intermediate with at least one intermediate-converting enzyme, wherein said intermediate is substantially all converted by said intermediate enzyme to said end-product, and wherein the presence of said substrate does not inhibit the further production of said end-product.

24. The method of Claim 23, wherein said intermediate-converting enzyme is a microbial enzyme.

25. The method of Claim 23, wherein said intermediate-converting microbial enzyme is secreted by a microorganism in contact with said intermediate.

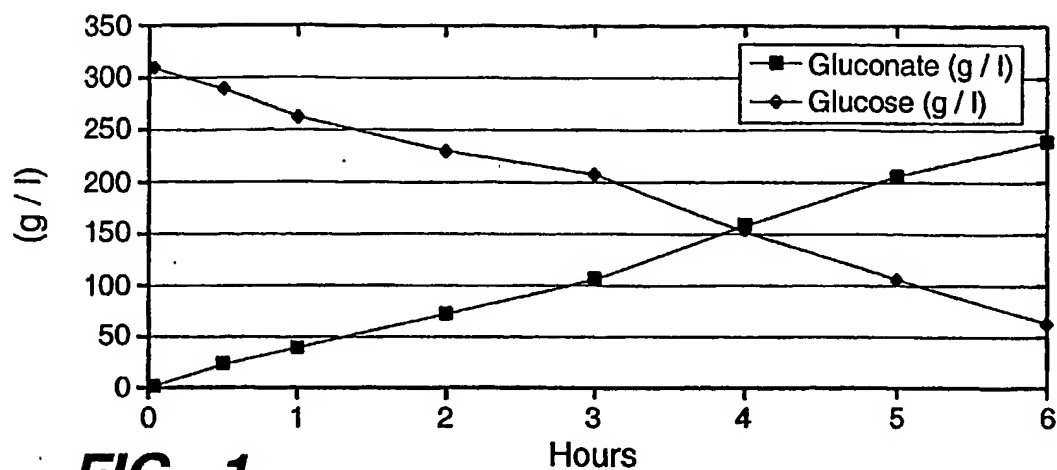
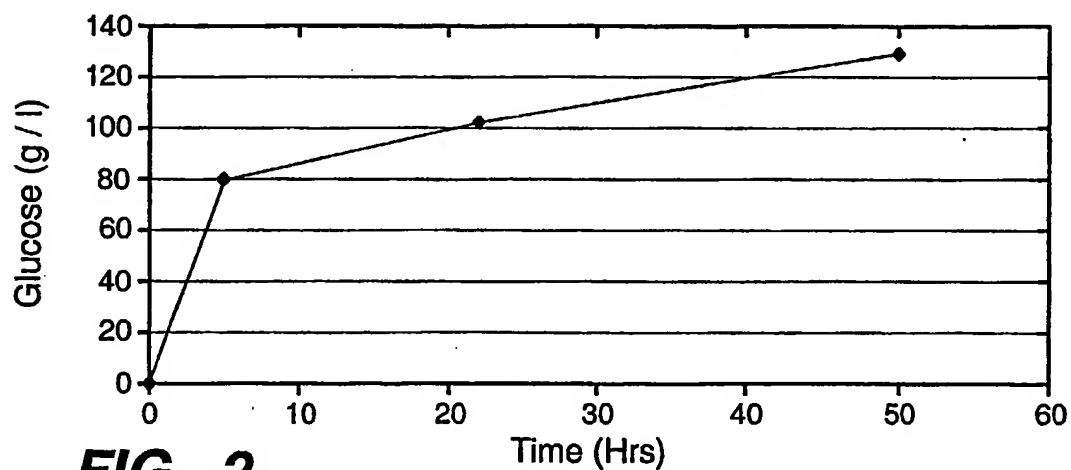
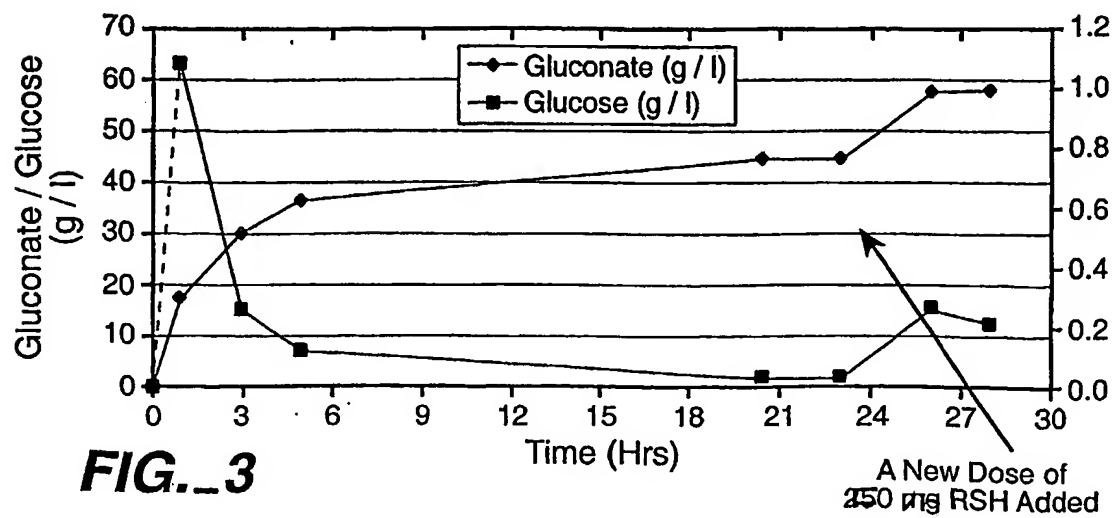
26. The method of Claim 23, wherein said substrate-converting enzyme is a microbial enzyme.

27. The method of Claim 23, wherein said substrate-converting microbial enzyme is produced is secreted by a microorganism in contact with said substrate.

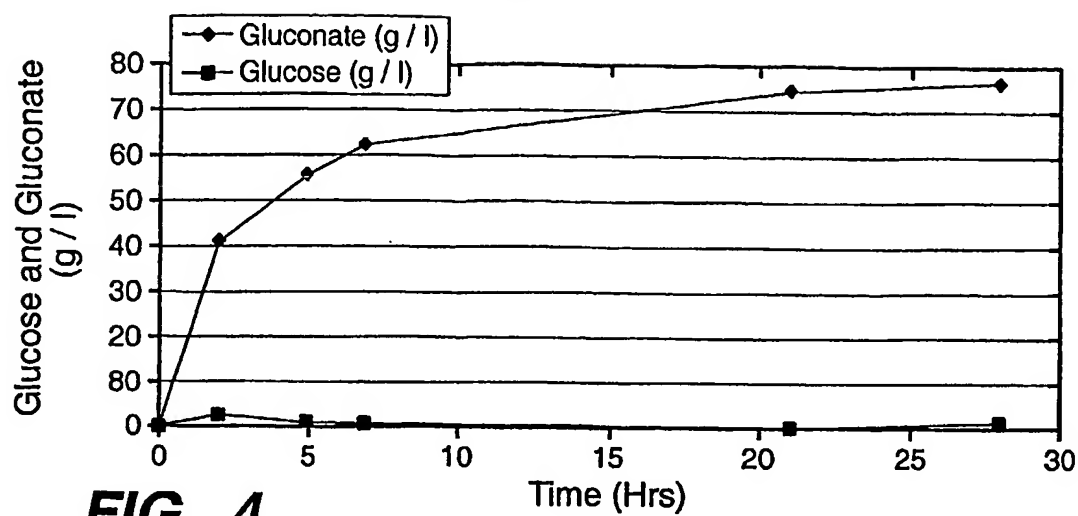
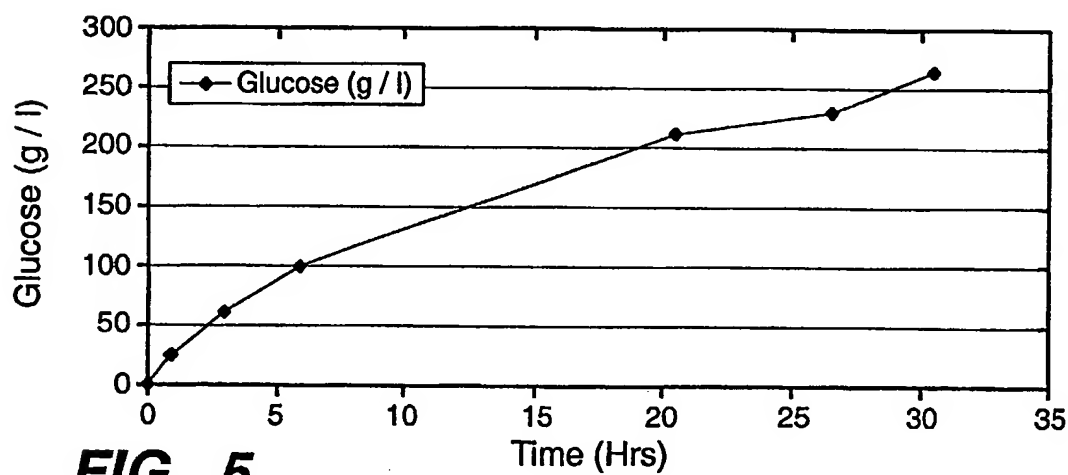
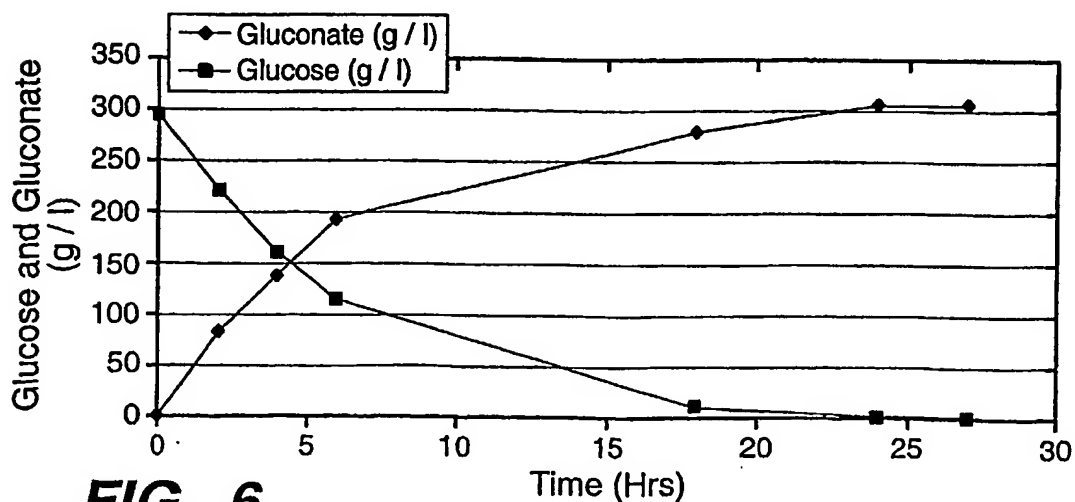
28. The method of Claim 23, wherein said intermediate-converting enzyme and said substrate-converting enzyme are produced by microorganisms of the same species.

29. The method of Claim 23, wherein said intermediate-converting enzyme and said substrate-converting enzyme are produced by microorganisms of the different species.

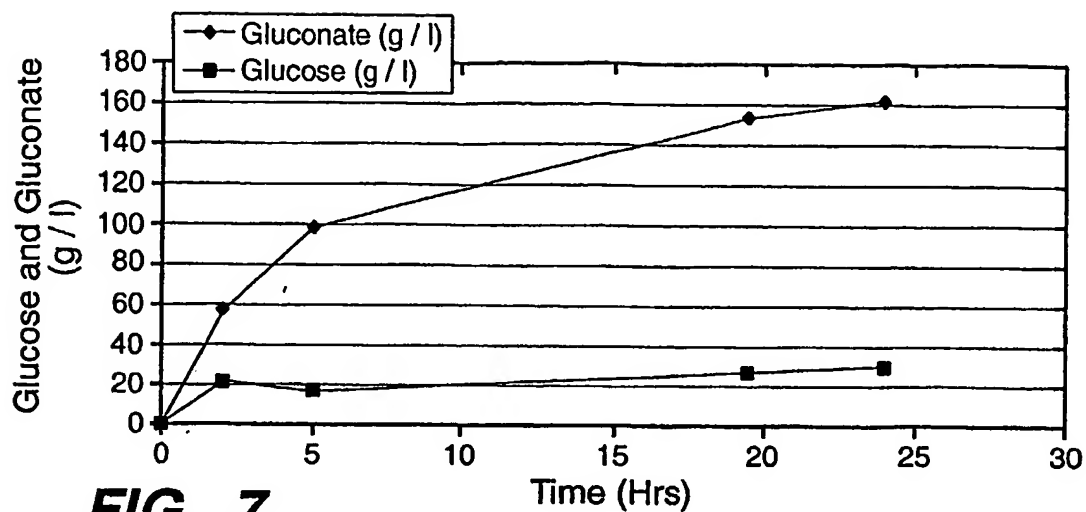
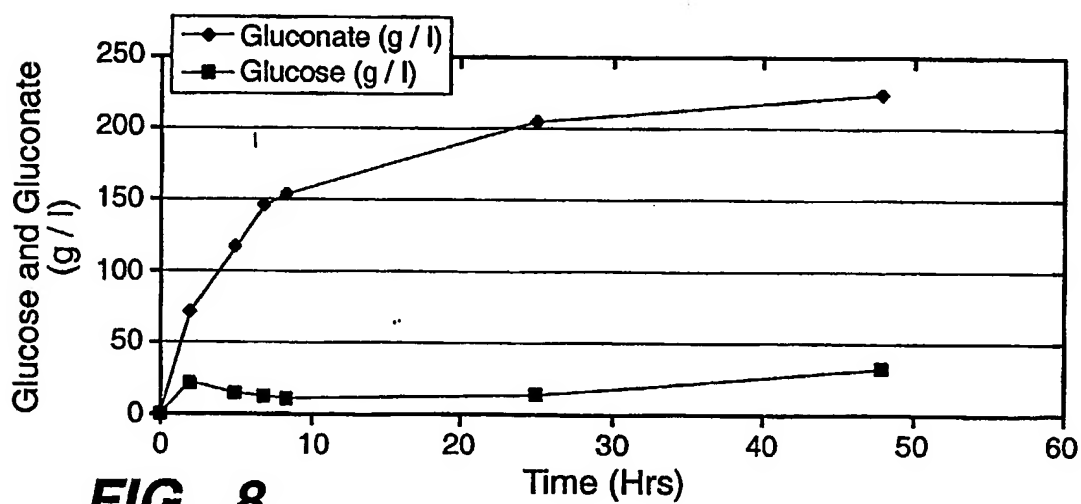
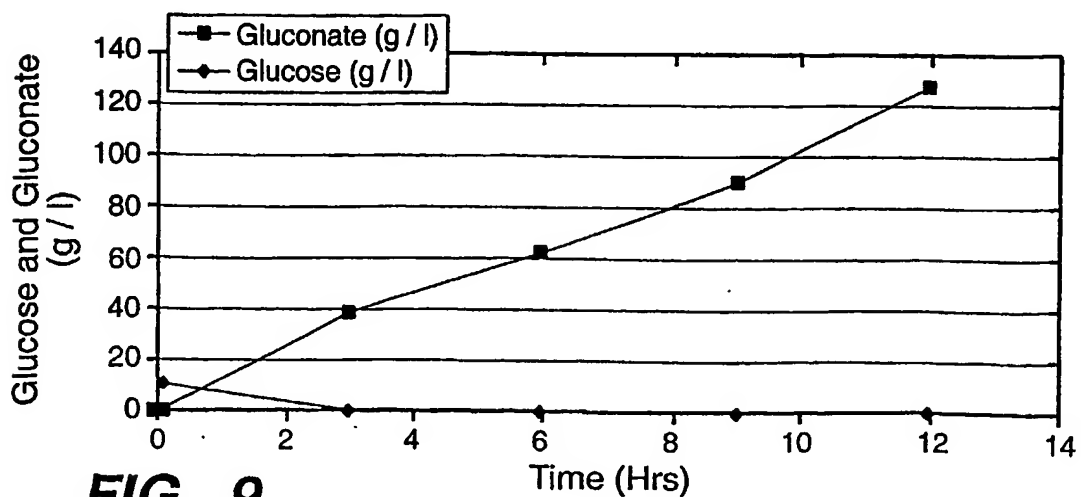
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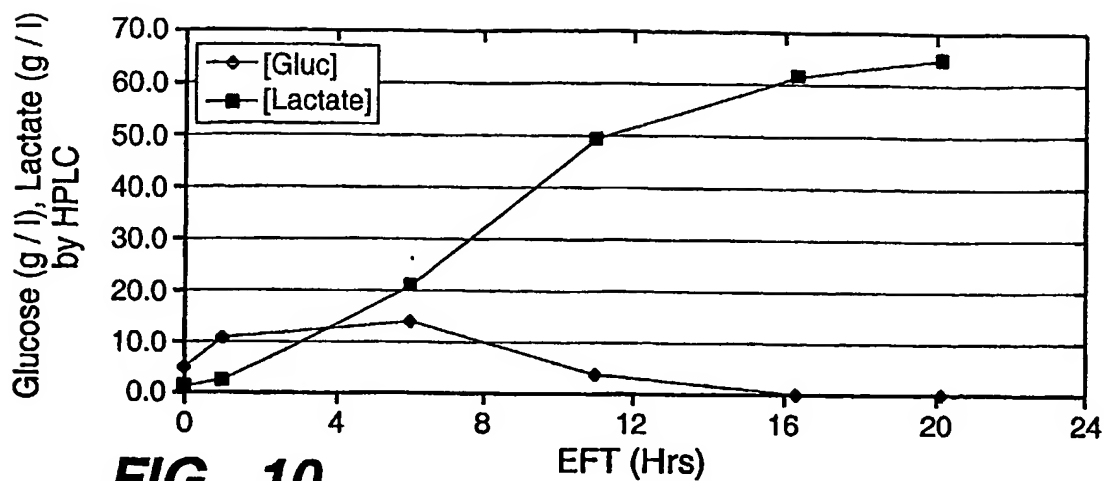
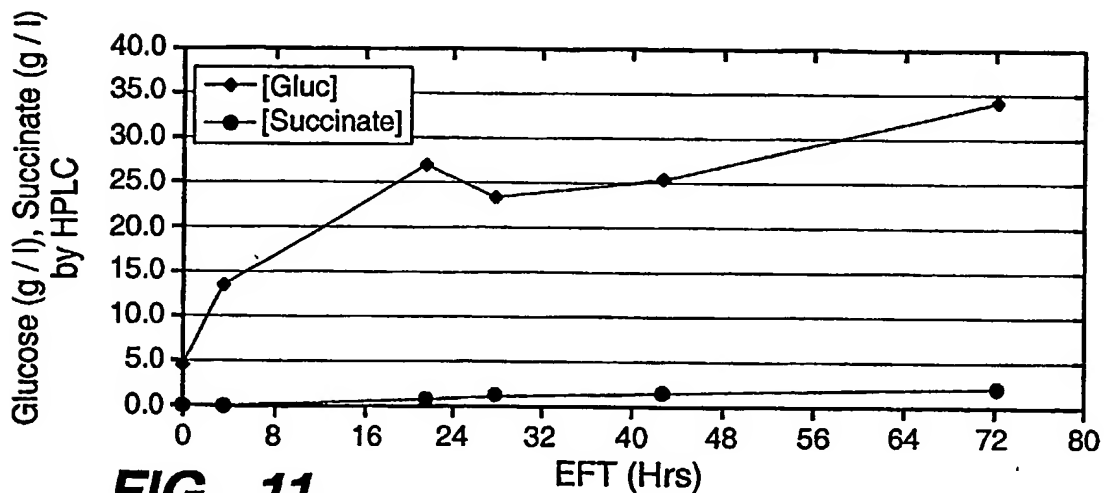
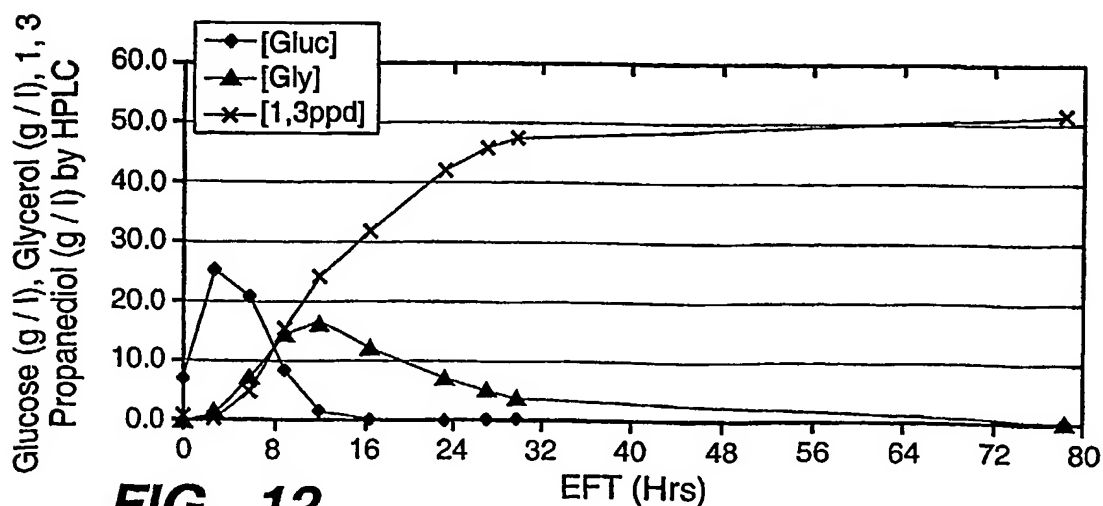
**FIG. 1****FIG. 2****FIG. 3**

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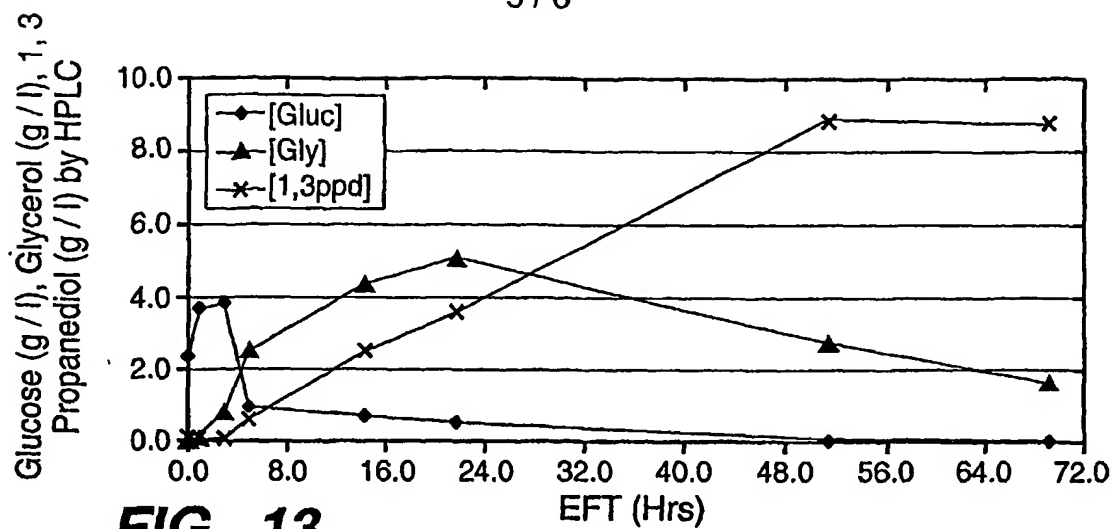
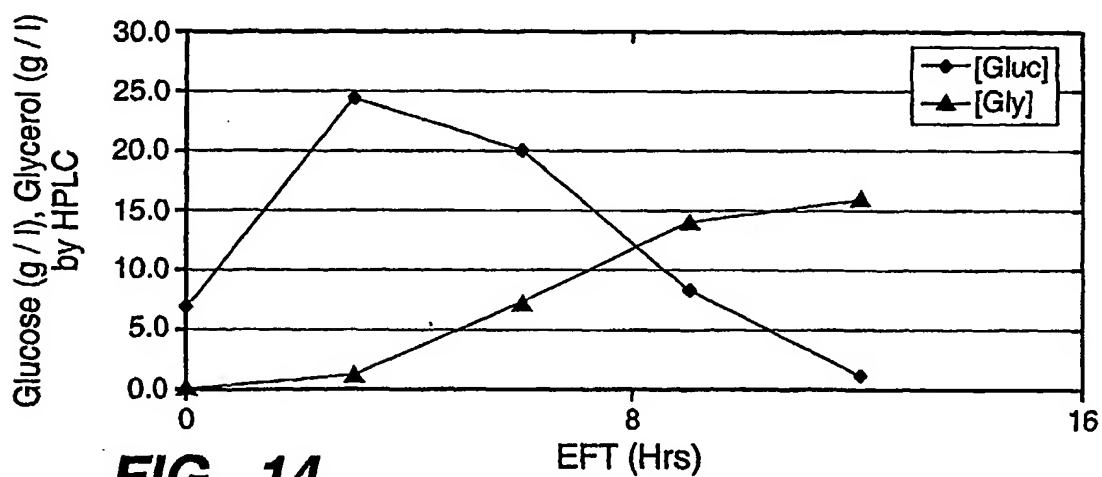
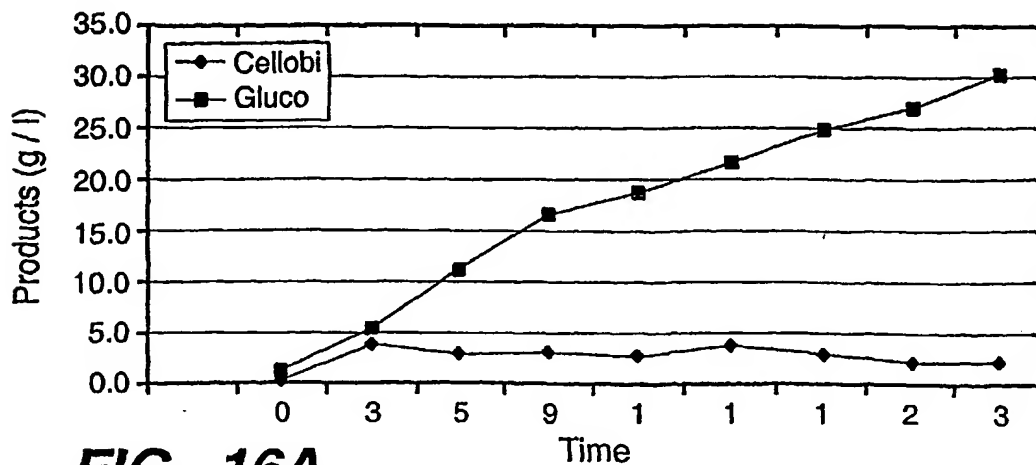
**FIG. 4****FIG. 5****FIG. 6**

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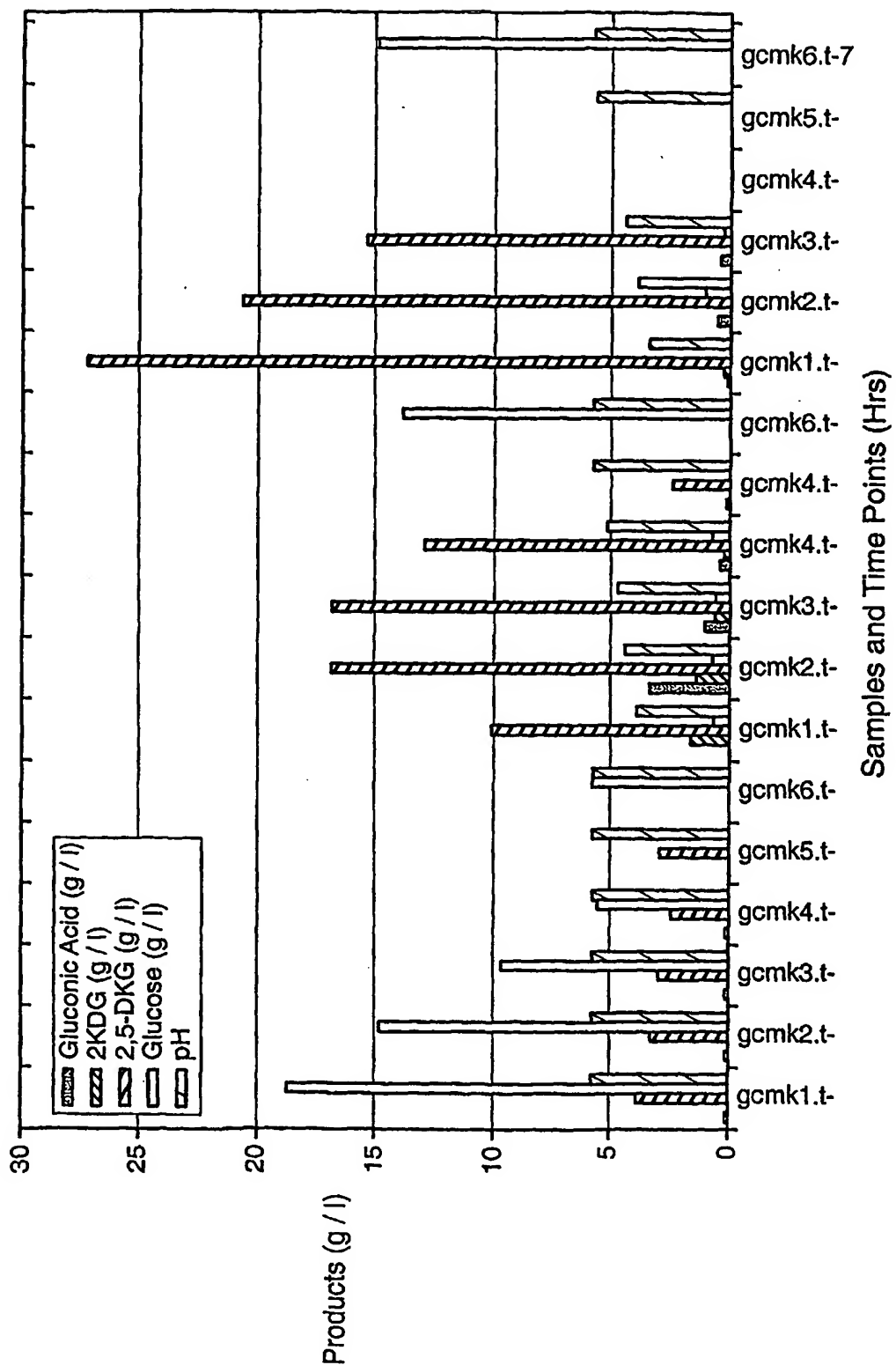
**FIG. 7****FIG. 8****FIG. 9**

**FIG. 10****FIG. 11****FIG. 12**

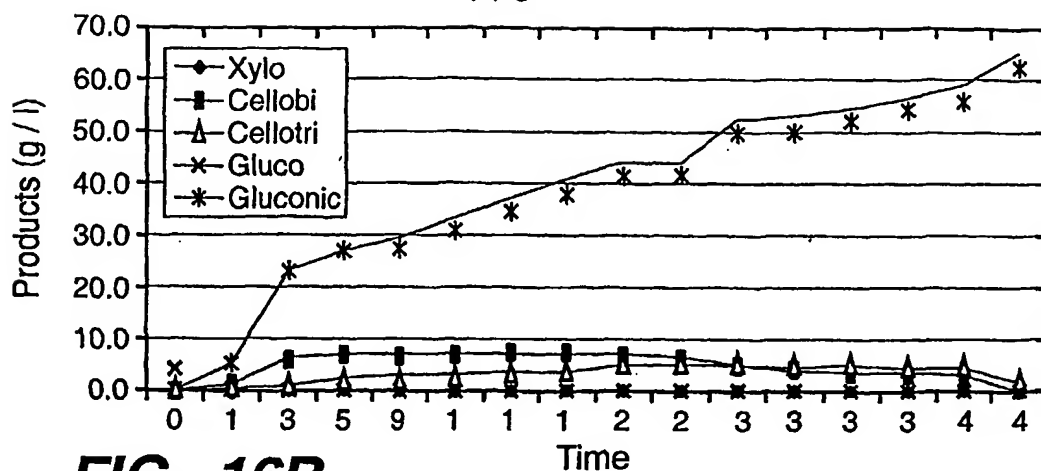
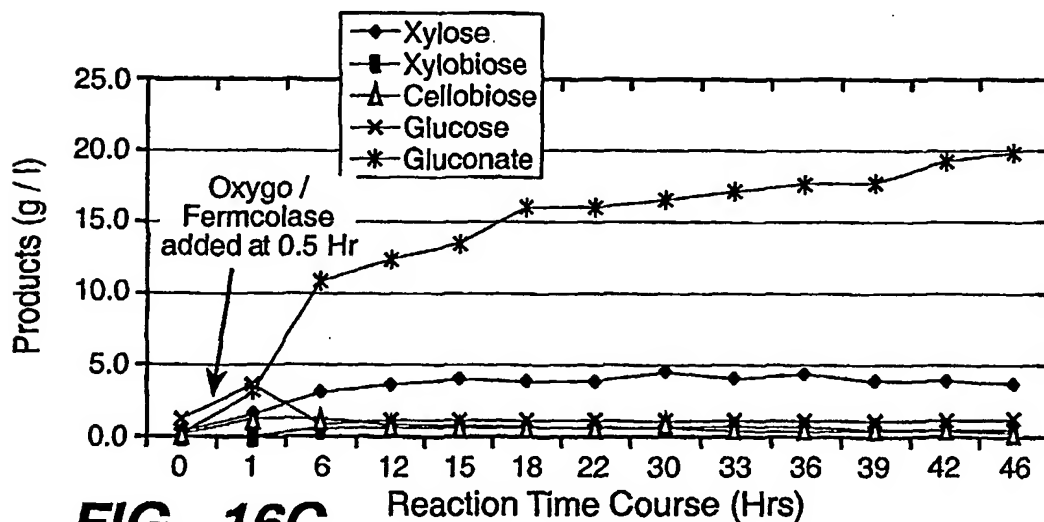
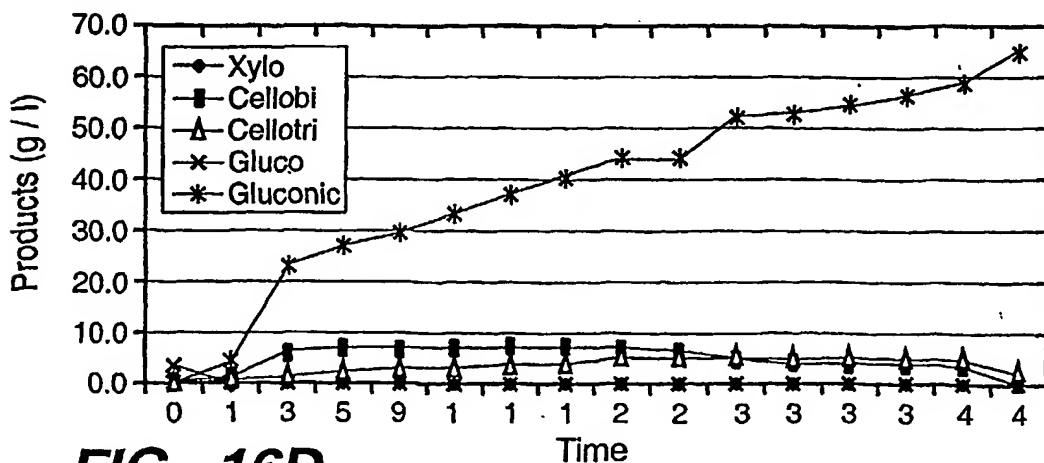
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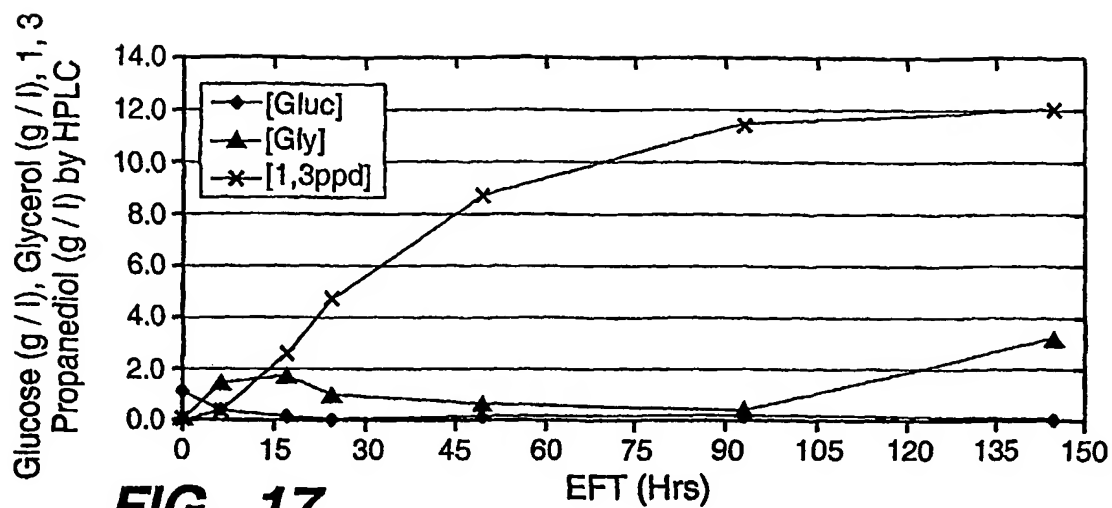
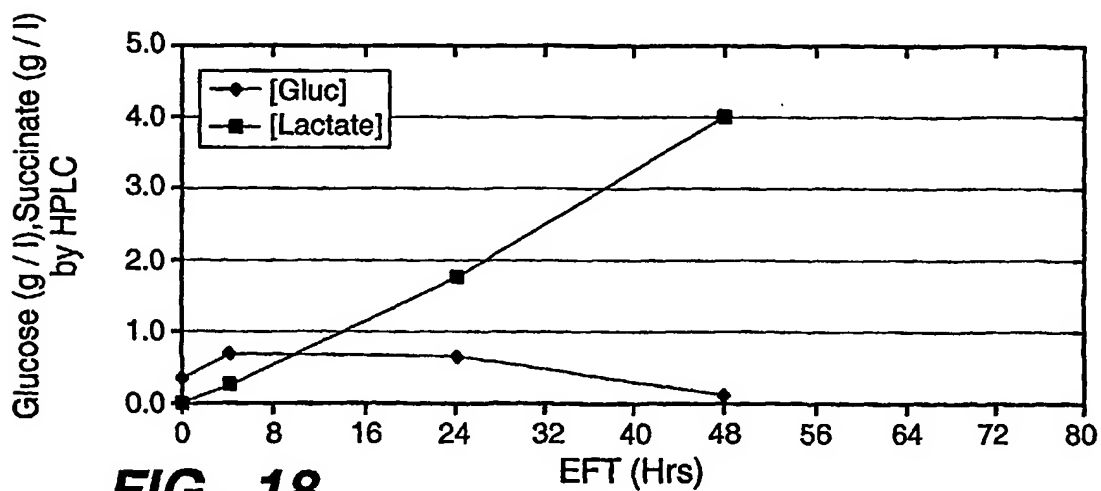
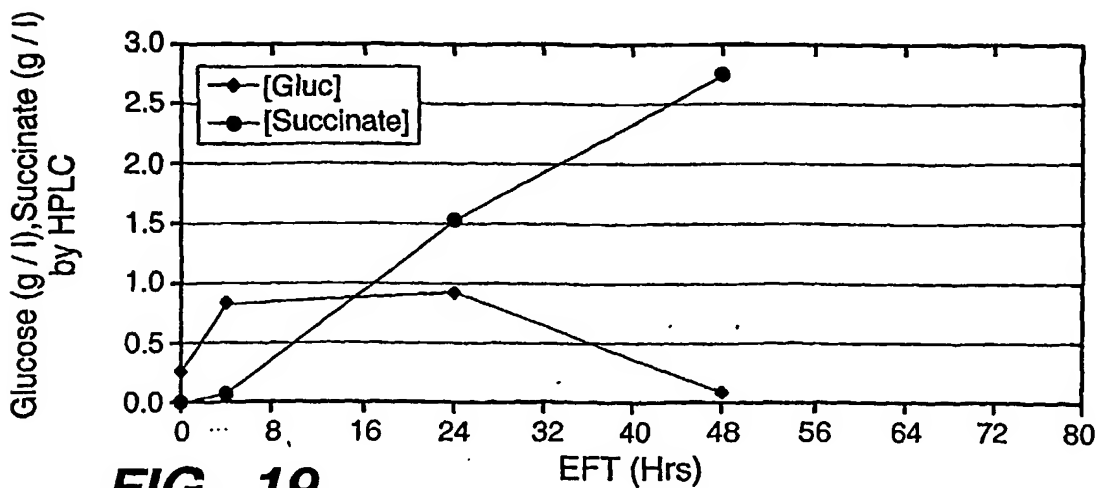
**FIG.\_13****FIG.\_14****FIG.\_16A**

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**FIG. 15**

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**FIG. 16B****FIG. 16C****FIG. 16D**

**FIG.\_17****FIG.\_18****FIG.\_19**

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WO 03/068976 A2

(54) Title: STARCH PROCESS

(57) Abstract: The present invention relates to a process for enzymatic hydrolysis of granular starch into a soluble starch hydrolysate at a temperature below the initial gelatinization temperature of said granular starch.

## STARCH PROCESS

### FIELD OF THE INVENTION

The present invention relates to a one step process for hydrolysis of granular starch into a soluble starch hydrolysate at a temperature below the initial gelatinization temperature of said granular starch.

### BACKGROUND OF THE INVENTION

A large number of processes have been described for converting starch to starch hydrolysates, such as maltose, glucose or specialty syrups, either for use as sweeteners or as precursors for other saccharides such as fructose. Glucose may also be fermented to ethanol or other fermentation products.

Starch is a high molecular-weight polymer consisting of chains of glucose units. It usually consists of about 80% amylopectin and 20% amylose. Amylopectin is a branched polysaccharide in which linear chains of alpha-1,4 D-glucose residues are joined by alpha-1,6 glucosidic linkages.

Amylose is a linear polysaccharide built up of D-glucopyranose units linked together by alpha-1,4 glucosidic linkages. In the case of converting starch into a soluble starch hydrolysate, the starch is depolymerized. The conventional depolymerization process consists of a gelatinization step and two consecutive process steps, namely a liquefaction process and a saccharification process.

Granular starch consists of microscopic granules, which are insoluble in water at room temperature. When an aqueous starch slurry is heated, the granules swell and eventually burst, dispersing the starch molecules into the solution. During this "gelatinization" process there is a dramatic increase in viscosity. As the solids level is 30-40% in a typical industrial process, the starch has to be thinned or "liquefied" so that it can be handled. This reduction in viscosity is today mostly obtained by enzymatic degradation. During the liquefaction step, the long-chained starch is degraded into smaller branched and linear units (maltodextrins) by an alpha-amylase. The liquefaction process is typically carried out at about 105-110°C for about 5 to 10 minutes followed by about 1-2 hours at about 95°C. The temperature is then lowered to 60°C, a glucoamylase or a beta-amylase and optionally a debranching enzyme, such as an isoamylase or a pullulanase are added, and the saccharification process proceeds for about 24 to 72 hours.

It will be apparent from the above discussion that the conventional starch conversion process is very energy consuming due to the different requirements in terms of temperature during the various steps. It is thus desirable to be able to select the enzymes used in the process so that the overall process can be performed without having to gelatinize the starch.

Such processes are the subject for the patents US4591560, US4727026 and US4009074 and EP0171218.

5 The present invention relates to a one-step process for converting granular starch into soluble starch hydrolysate at a temperature below initial gelatinization temperature of the starch.

## SUMMARY OF THE INVENTION

10 In a first aspect the invention provides a one step process for producing a soluble starch hydrolysate, the process comprising subjecting a aqueous granular starch slurry at a temperature below the initial gelatinization temperature of said granular starch to the simultaneous action of the following enzyme activities, a first enzyme which is a member of the Glycoside Hydrolase Family 13, has alpha-1.4-glucosidic hydrolysis activity and comprises a Carbohydrate-Binding Module of Family 20, and a second enzyme which is a fungal alpha-amylase (EC 3.2.1.1), a beta-amylase (E.C. 3.2.1.2), or an glucoamylase (E.C.3.2.1.3).

15 In a second aspect the invention provides a process for production of high fructose starch-based syrup (HFSS), the process comprising producing a soluble starch hydrolysate by the process of the first aspect of the invention, and further comprising a step for conversion of the soluble starch hydrolysate into a of high fructose starch-based syrup (HFSS).

20 In a third aspect the invention provides a process for production of fuel or potable ethanol; comprising producing a soluble starch hydrolysate by the process of the first aspect of the invention, and further comprising a step for fermentation of the soluble starch hydrolysate into ethanol, wherein the fermentation step is carried out simultaneously or separately/sequential to the hydrolysis of the granular starch.

## DETAILED DESCRIPTION OF THE INVENTION

### 25 Definitions

The term "granular starch" is understood as raw uncooked starch, i.e. starch that has not been subjected to a gelatinization. Starch is formed in plants as tiny granules insoluble in water. These granules are preserved in starches at temperatures below the initial gelatinization temperature. When put in cold water, the grains may absorb a small amount of the liquid. Up to 50°C to 70°C the swelling is reversible, the degree of reversibility being dependent upon the particular starch. With higher temperatures an irreversible swelling called gelatinization begins.

35 The term "initial gelatinization temperature" is understood as the lowest temperature at which gelatinization of the starch commences. Starch begins to gelatinize between 60°C and 70°C, the exact temperature dependent on the specific starch. The initial gelatinization

temperature depends on the source of the starch to be processed. The initial gelatinization temperature for wheat starch is approximately 52°C, for potato starch approximately 56°C, and for corn starch approximately 62°C. However, the quality of the starch initial may vary according to the particular variety of the plant species as well as with the growth conditions and therefore initial gelatinization temperature should be determined for each individual starch lot.

The term "soluble starch hydrolysate" is understood as the soluble products of the processes of the invention and may comprise mono- di-, and oligosaccharides, such as glucose, maltose, maltodextrins, cyclodextrins and any mixture of these. Preferably at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97% or 98% of the dry solids of the granular starch is converted into a soluble starch hydrolysate.

The term "Speciality Syrups", is an in the art recognised term and is characterised according to DE and carbohydrate spectrum (See the article "New Speciality Glucose Syrups", p. 50+, in the textbook "Molecular Structure and Function of Food Carbohydrate", Edited by G.G. Birch and L.F. Green, Applied Science Publishers LTD., London). Typically Speciality Syrups have a DE in the range from 35 to 45.

The "Glycoside Hydrolase Family 13" is in the context of this invention defined as the group of hydrolases comprising a catalytic domain having a (beta/alpha)<sub>8</sub> or TIM barrel structure and acting on starch and related substrates through an alpha-retaining reacting mechanism (Koshland, 1953, Biol.Rev.Camp.Philos.Soc 28, 416-436).

The enzymes having "alpha-1,4-glucosidic hydrolysis activity" is in the context of this invention defined as comprising the group of enzymes which catalyze the hydrolysis and/or synthesis of alpha-1,4-glucosidic bonds as defined by Takata (Takata et al, 1992, J. Biol. Chem. 267, 18447-18452) and by Koshland (Koshland, 1953, Biol.Rev. Camp. Philos. Soc 28, 416-436).

The "Carbohydrate-Binding Module of Family 20" or a CBM-20 module is in the context of this invention defined as a sequence of approximately 100 amino acids having at least 45% homology to the Carbohydrate-Binding Module (CBM) of the polypeptide disclosed in figure 1 by Joergensen et al (1997) in Biotechnol. Lett. 19:1027-1031. The CBM comprises the last 102 amino acids of the polypeptide, i.e. the subsequence from amino acid 582 to amino acid 683.

Enzymes which; (a) are members of the Glycoside Hydrolase Family 13; (b) have alpha-1,4-glucosidic hydrolysis activity and (c) comprise a Carbohydrate-Binding Module of Family 20, and are specifically contemplated for this invention comprise the enzymes classified as EC 2.4.1.19, the cyclodextrin glucanotransferases, and EC 3.2.1.133, the maltogenic alpha-amylases, and selected members of 3.2.1.1 the alpha-amylases, and 3.2.1.60, the maltotetraose-forming amylases.

The "hydrolysis activity" of CGTases and maltogenic alpha-amylases is determined by measuring the increase in reducing power during incubation with starch according to Wind, R.D. et al 1995 in Appl. Environ. Microbiol.61:1257-1265. Reducing sugar concentrations is measured with the dinitrosalicylic acid method according to Bemfield (Bemfield, P. 1955. Amylases alpha and beta. Methods Enzymol. 1:149-158), with a few modifications. Diluted enzyme is incubated for an appropriate period of time with 1% (wt/v) soluble starch (Paselli SA2 starch from Avebe, The Netherlands or alternatively soluble starch from Merck) in a 10 mM sodium citrate (pH 5.9) buffer at 60°C. One unit of hydrolysis activity is defined as the amount of enzyme producing 1 micro mol of maltose per minute under standard conditions.

The polypeptide "homology" referred to in this disclosure is understood as the degree of identity between two sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-453. The following settings for polypeptide sequence comparison are used: GAP creation penalty of 3.0 and GAP extension penalty of 0.1.

#### Cyclodextrin glucanotransferases (CGTases)

A particular enzyme to be used as a first enzyme in the processes of the invention may be a cyclomaltodextrin glucanotransferase (E.C. 2.4.1.19). Cyclomaltodextrin glucanotransferase, also designated cyclodextrin glucanotransferase or cyclodextrin glycosyltransferase, in the following termed CGTase, catalyses the conversion of starch and similar substrates into cyclomaltodextrins via an intramolecular transglycosylation reaction, thereby forming cyclomaltodextrins of various sizes. Most CGTases have both transglycosylation activity and starch-degrading activity. Contemplated CGTases are preferably of microbial origin, and most preferably of bacterial origin. Specifically contemplated CGTases include the CGTases having 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or even 90% homology to the sequence shown as amino acids 1 to 679 of SEQ ID NO:2 in WO02/06508, the CGTases having 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or even 90% homology to the amino acid sequence of the polypeptide disclosed in Joergensen et al, 1997 in figure 1 in Biotechnol. Lett. 19:1027-1031, and the CGTases described in US5278059 and US5545587. Preferably the CGTase to be applied as a first enzyme of the process has a hydrolysis activity of at least 3.5, preferably at least 4, 4.5, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, or most preferably at least 23 micro mol per min/mg. CGTases may be added in amounts of 0.01-100.0 NU/g DS, preferably from 0.2-50.0 NU/g DS, preferably 10.0-20.0 NU/g DS.

### Maltogenic alpha-amylase

Another particular enzyme to be used as a first enzyme in the processes of the invention is a maltogenic alpha-amylase (E.C. 3.2.1.133). Maltogenic alpha-amylases (glucan 1,4-alpha-maltohydrolase) are able to hydrolyse amylose and amylopectin to maltose in the alpha-configuration. Furthermore, a maltogenic alpha-amylase is able to hydrolyse maltotriose as well as cyclodextrins. Specifically contemplated maltogenic alpha-amylases may be derived from *Bacillus* sp., preferably from *Bacillus stearothermophilus*, most preferably from *Bacillus stearothermophilus* C599 such as the one described in EP120.693. This particular maltogenic alpha-amylase has the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1 in US6162628. A preferred maltogenic alpha-amylase has an amino acid sequence having at least 70% identity to amino acids 1-686 of SEQ ID NO:1 in US6162628, preferably at least 80%, at least 85%, at least 90%, at least 92%, at least 95%, at least 96%, at least 97%, at least 98%, or particularly at least 99%. Most preferred variants of the maltogenic alpha-amylase comprise the variants disclosed in WO99/43794.

The maltogenic alpha-amylase having the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1 in US6162628 has a hydrolysis activity of 714. Preferably the maltogenic alpha-amylase to be applied as a first enzyme of the process has a hydrolysis activity of at least 3.5, preferably at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 100, 200, 300, 400, 500, 600, or most preferably at least 700 micro mol per min/mg.

Maltogenic alpha-amylases may be added in amounts of 0.01-40.0 MANU/g DS, preferably from 0.02-10 MANU/g DS, preferably 0.05-5.0 MANU/g DS.

### Fungal alpha-amylase

A particular enzyme to be used as a second enzyme in the processes of the invention is a fungal alpha-amylase (EC 3.2.1.1), such as a fungamyl-like alpha-amylase. In the present disclosure, the term "fungamyl-like alpha-amylase" indicates an alpha-amylase which exhibits a high homology, i.e. more than 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or even 90% homology to the amino acid sequence shown in SEQ ID No. 10 in WO96/23874. Fungal alpha-amylases may be added in an amount of 0.001-1.0 AFAU/g DS, preferably from 0.002-0.5 AFAU/g DS, preferably 0.02-0.1 AFAU/g DS.

### Beta-amylase

Another particular enzyme to be used as a second enzyme in the processes of the invention may be a beta-amylase (E.C 3.2.1.2). Beta-amylase is the name traditionally given to exo-acting maltogenic amylases, which catalyze the hydrolysis of 1,4-alpha-glucosidic linkages in amylose, amylopectin and related glucose polymers.

Beta-amylases have been isolated from various plants and microorganisms (W.M. Fogarty and C.T. Kelly, Progress in Industrial Microbiology, vol. 15, pp. 112-115, 1979). These

beta-amylases are characterized by having optimum temperatures in the range from 40°C to 65°C and optimum pH in the range from 4.5 to 7.0. Contemplated beta-amylase include the beta-amylase from barley Spezyme® BBA 1500, Spezyme® DBA and Optimalt™ ME, Optimalt™ BBA from Genencor int as well as Novozym™ WBA from Novozymes A/S.

## 5 Glucoamylase

A further particular enzyme to be used as a second enzyme in the processes of the invention may also be a glucoamylase (E.C.3.2.1.3) derived from a microorganism or a plant. Preferred is glucoamylases of fungal or bacterial origin selected from the group consisting of *Aspergillus* glucoamylases, in particular *A. niger* G1 or G2 glucoamylase (Boel et al. (1984),  
 10 EMBO J. 3 (5), p. 1097-1102), or variants thereof, such as disclosed in WO92/00381 and WO00/04136; the *A. awamori* glucoamylase (WO84/02921), *A. oryzae* (Agric. Biol. Chem. (1991), 55 (4), p. 941-949), or variants or fragments thereof.

Other contemplated *Aspergillus* glucoamylase variants include variants to enhance the thermal stability: G137A and G139A (Chen et al. (1996), *Prot. Engng.* 9, 499-505); D257E  
 15 and D293E/Q (Chen et al. (1995), *Prot. Engng.* 8, 575-582); N182 (Chen et al. (1994), *Biochem. J.* 301, 275-281); disulphide bonds, A246C (Fierobe et al. (1996), *Biochemistry*, 35, 8698-8704; and introduction of Pro residues in position A435 and S436 (Li et al. (1997), *Protein Engng.* 10, 1199-1204. Furthermore Clark Ford presented a paper on Oct 17, 1997, ENZYME ENGINEERING 14, Beijing/China Oct 12-17, 97, Abstract book p. 0-61. The abstract  
 20 suggests mutations in positions G137A, N20C/A27C, and S30P in an *Aspergillus awamori* glucoamylase to improve the thermal stability. Other contemplated glucoamylases include *Talaromyces* glucoamylases, in particular derived from *Talaromyces emersonii* (WO99/28448), *Talaromyces leycettanus* (US patent no. Re.32,153), *Talaromyces duponti*, *Talaromyces thermophilus* (US patent no. 4,587,215). Bacterial glucoamylases contemplated  
 25 include glucoamylases from the genus *Clostridium*, in particular *C. thermoamylolyticum* (EP135,138), and *C. thermohydrosulfuricum* (WO86/01831). Preferred glucoamylases include the glucoamylases derived from *Aspergillus oryzae*, such as a glucoamylase having 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or even 90% homology to the amino acid sequence shown in SEQ ID NO:2 in WO00/04136. Also contemplated are the commercial products AMG  
 30 200L; AMG 300 L; SAN™ SUPER and AMG™ E (from Novozymes); OPTIDEX™ 300 (from Genencor Int.); AMIGASE™ and AMIGASE™ PLUS (from DSM); G-ZYME™ G900 (from Enzyme Bio-Systems); G-ZYME™ G990 ZR (*A. niger* glucoamylase and low protease content).

Glucoamylases may be added in an amount of 0.02-2.0 AGU/g DS, preferably 0.1-1.0  
 35 AGU/g DS, such as 0.2 AGU/g DS.

## Additional enzymes.

The processes of the invention may also be carried out in the presence of a third enzyme. A particular third enzyme may be a *Bacillus* alpha-amylase (often referred to as

"Termamyl-like alpha-amylases"). Well-known Termamyl-like alpha-amylases include alpha-amylase derived from a strain of *B. licheniformis* (commercially available as Termamyl), *B. amyloliquefaciens*, and *B. stearothermophilus* alpha-amylase. Other Termamyl-like alpha-amylases include alpha-amylase derived from a strain of the *Bacillus* sp. NCIB 12289, NCIB 12512, NCIB 12513 or DSM 9375, all of which are described in detail in WO95/26397, and the alpha-amylase described by Tsukamoto et al., Biochemical and Biophysical Research Communications, 151 (1988), pp. 25-31. In the context of the present invention a Termamyl-like alpha-amylase is an alpha-amylase as defined in WO99/19467 on page 3, line 18 to page 6, line 27. Contemplated variants and hybrids are described in WO96/23874, WO97/41213, and WO99/19467. Specifically contemplated is a recombinant *B. stearothermophilus* alpha-amylase variant with the mutations: I181\* + G182\* + N193F. *Bacillus* alpha-amylases may be added in effective amounts well known to the person skilled in the art.

Another particular third enzyme of the process may be a debranching enzyme, such as an isoamylase (E.C. 3.2.1.68) or a pullulanases (E.C. 3.2.1.41). Isoamylase hydrolyses alpha-1,6-D-glucosidic branch linkages in amylopectin and beta-limit dextrins and can be distinguished from pullulanases by the inability of isoamylase to attack pullulan, and by the limited action on alpha-limit dextrins. Debranching enzyme may be added in effective amounts well known to the person skilled in the art.

#### Embodiments of the invention

The starch slurry to be subjected to the processes of the invention may have 20-55% dry solids granular starch, preferably 25-40% dry solids granular starch, more preferably 30-35% dry solids granular starch.

After being subjected to the process of the first aspect of the invention at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or preferably 99% of the dry solids of the granular starch is converted into a soluble starch hydrolysate.

According to the invention the processes of the first and second aspect is conducted at a temperature below the initial gelatinization temperature. Preferably the temperature at which the processes are conducted is at least 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, 41°C, 42°C, 43°C, 44°C, 45°C, 46°C, 47°C, 48°C, 49°C, 50°C, 51°C, 52°C, 53°C, 54°C, 55°C, 56°C, 57°C, 58°C, 59°C, or preferably at least 60°C.

The pH at which the process of the first aspect of the invention is conducted may in be in the range of 3.0 to 7.0, preferably from 3.5 to 6.0, or more preferably from 4.0-5.0.

The exact composition of the products of the process of the first aspect of the invention, the soluble starch hydrolysate, depends on the combination of enzymes applied as well as the type of granular starch processed. Preferably the soluble hydrolysate is maltose with a purity of at least 85%, 90%, 95.0%, 95.5%, 96.0%, 96.5%, 97.0%, 97.5%, 98.0%, 98.5, 99.0% or 99.5%. Even more preferably the soluble starch hydrolysate is glucose, and most

preferably the starch hydrolysate has a DX (glucose percent of total solubilised dry solids) of at least 94.5%, 95.0%, 95.5%, 96.0%, 96.5%, 97.0%, 97.5%, 98.0%, 98.5, 99.0% or 99.5%. Equally contemplated, however, is the process wherein the product of the process of the invention, the soluble starch hydrolysate, is a speciality syrup, such as a speciality syrup  
5 containing a mixture of glucose, maltose, DP3 and DPn for use in the manufacture of ice creams, cakes, candies, canned fruit.

The granular starch to be processed in the processes of the invention may in particular be obtained from tubers, roots, stems, legumes, cereals or whole grain. More specifically the granular starch may be obtained from corns, cobs, wheat, barley, rye, milo,  
10 sago, cassava, tapioca, sorghum, rice, peas, bean, banana or potatoes. Specially contemplated are both waxy and non-waxy types of corn and barley. The granular starch to be processed may be a highly refined starch quality, preferably more than 90%, 95%, 97% or 99.5 % pure or it may be a more crude starch containing material comprising milled whole grain including non-starch fractions such as germ residues and fibres. The raw material, such  
15 as whole grain, is milled in order to open up the structure and allowing for further processing. Two milling processes are preferred according to the invention: wet and dry milling. In dry milling the whole kernel is milled and used. Wet milling gives a good separation of germ and meal (starch granules and protein) and is with a few exceptions applied at locations where the starch hydrolysate is used in production of syrups. Both dry and wet milling is well known in  
20 the art of starch processing and are equally contemplated for the processes of the invention. The process of the first aspect of the invention may be conducted in an ultrafiltration system where the retentate is held under recirculation in presence of enzymes, raw starch and water and where the permeate is the soluble starch hydrolysate. Equally contemplated is the process conducted in a continuous membrane reactor with ultrafiltration membranes and  
25 where the retentate is held under recirculation in presence of enzymes, raw starch and water and where the permeate is the soluble starch hydrolysate. Also contemplated is the process conducted in a continuous membrane reactor with microfiltration membranes and where the retentate is held under recirculation in presence of enzymes, raw starch and water and where the permeate is the soluble starch hydrolysate.

30 In the process of the second aspect of the invention the soluble starch hydrolysate of the process of the first aspect of the invention is subjected to conversion into high fructose starch-based syrup (HFSS), such as high fructose corn syrup (HFCS). This conversion is preferably achieved using a glucose isomerase, and more preferably by an immobilized glucose isomerase supported on a solid support. Contemplated isomerases comprises the  
35 commercial products Sweetzyme™ IT from Novozymes A/S; G -zyme™ IMGI and G-zyme™ G993, Ketomax™ and G-zyme™ G993 from Rhodia, G-zyme™ G993 liquid and GenSweet™ IGI from Genemcor Int.

In the process of the third aspect of the invention the soluble starch hydrolysate of the process of the first aspect of the invention is used for production of fuel or potable ethanol. In the process of the third aspect the fermentation may be carried out simultaneously or separately/sequential to the hydrolysis of the granular starch slurry. When the fermentation is performed simultaneous to the hydrolysis the temperature is preferably between 30°C and 35°C, and more preferably between 31°C and 34°C. The process of the third aspect of the invention may be conducted in an ultrafiltration system where the retentate is held under recirculation in presence of enzymes, raw starch, yeast, yeast nutrients and water and where the permeate is an ethanol containing liquid. Equally contemplated is the process conducted in a continuous membrane reactor with ultrafiltration membranes and where the retentate is held under recirculation in presence of enzymes, raw starch, yeast, yeast nutrients and water and where the permeate is an ethanol containing liquid.

## MATERIALS AND METHODS

### Alpha-amylase activity (KNU)

The amylolytic activity may be determined using potato starch as substrate. This method is based on the break-down of modified potato starch by the enzyme, and the reaction is followed by mixing samples of the starch/enzyme solution with an iodine solution. Initially, a blackish-blue colour is formed, but during the break-down of the starch the blue colour gets weaker and gradually turns into a reddish-brown, which is compared to a coloured glass standard.

One Kilo Novo alpha amylase Unit (KNU) is defined as the amount of enzyme which, under standard conditions (i.e. at 37°C +/- 0.05; 0.0003 M  $\text{Ca}^{2+}$ ; and pH 5.6) dextrinizes 5.26 g starch dry substance Merck Amylum soluble.

A folder AF 9/6 describing this analytical method in more detail is available upon request to Novozymes A/S, Denmark, which folder is hereby included by reference.

### CGTase activity (KNU)

The CGTase alpha-amylase activity is determined by a method employing Phadebas® tablets as substrate. Phadebas tablets (Phadebas® Amylase Test, supplied by Pharmacia Diagnostic) contain a cross-linked insoluble blue-colored starch polymer, which has been mixed with bovine serum albumin and a buffer substance.

For every single measurement one tablet is suspended in a tube containing 5 ml 50 mM Britton-Robinson buffer (50 mM acetic acid, 50 mM phosphoric acid, 50 mM boric acid, 0.1 mM  $\text{CaCl}_2$ , pH adjusted to the value of interest with NaOH). The test is performed in a water bath at the temperature of interest. The alpha-amylase to be tested is diluted in x ml of 50 mM Britton-Robinson buffer. 1 ml of this alpha-amylase solution is added to the 5 ml 50

mM Britton-Robinson buffer. The starch is hydrolyzed by the alpha-amylase giving soluble blue fragments. The absorbance of the resulting blue solution, measured spectrophotometrically at 620 nm, is a function of the alpha-amylase activity.

It is important that the measured 620 nm absorbance after 10 or 15 minutes of incubation (testing time) is in the range of 0.2 to 2.0 absorbance units at 620 nm. In this absorbance range there is linearity between activity and absorbance (Lambert-Beer law). The dilution of the enzyme must therefore be adjusted to fit this criterion. Under a specified set of conditions (temperature, pH, reaction time, buffer conditions) 1 mg of a given alpha-amylase will hydrolyze a certain amount of substrate and a blue colour will be produced. The colour intensity is measured at 620 nm. The measured absorbance is directly proportional to the specific activity (activity/mg of pure alpha-amylase protein) of the alpha-amylase in question under the given set of conditions.

A folder EAL-SM-0351 describing this analytical method in more detail is available upon request to Novozymes A/S, Denmark, which folder is hereby included by reference

#### 15 Maltogenic alpha-amylase activity (MANU)

One Maltogenic Amylase Novo Unit (MANU) is defined as the amount of enzyme which under standard will cleave one micro mol maltotriose per minute. The standard conditions are 10 mg/ml maltotriose, 37°C, pH 5.0, and 30 minutes reaction time. The formed glucose is converted by glucose dehydrogenase (GlucDH, Merck) to gluconolactone under formation of NADH, which is determined spectrophotometrically at 340 nm. A folder (EAL-SM-0203.01) describing this analytical method in more detail is available on request from Novozymes A/S, Denmark, which folder is hereby included by reference.

#### Glucoamylase activity (AGU)

The Novo Glucoamylase Unit (AGU) is defined as the amount of enzyme, which hydrolyzes 1 micromole maltose per minute at 37°C and pH 4.3.

The activity is determined as AGU/ml by a method modified after (AEL-SM-0131, available on request from Novozymes) using the Glucose GOD-Perid kit from Boehringer Mannheim, 124036. Standard: AMG-standard, batch 7-1195, 195 AGU/ml. 375 microL substrate (1% maltose in 50 mM Sodium acetate, pH 4.3) is incubated 5 minutes at 37°C. 25 microL enzyme diluted in sodium acetate is added. The reaction is stopped after 10 minutes by adding 100 microL 0.25 M NaOH. 20 microL is transferred to a 96 well microtitre plate and 200 microL GOD-Perid solution (124036, Boehringer Mannheim) is added. After 30 minutes at room temperature, the absorbance is measured at 650 nm and the activity calculated in AGU/ml from the AMG-standard. A folder (AEL-SM-0131) describing this analytical method in more detail is available on request from Novozymes A/S, Denmark, which folder is hereby included by reference.

Fungal alpha-amylase activity (FAU)

The alpha-amylase activity is measured in FAU (Fungal Alpha-Amylase Units). One (1) FAU is the amount of enzyme which under standard conditions (i.e. at 37°C and pH 4.7) breaks down 5260 mg solid starch (Amylum soluble, Merck) per hour. A folder AF 9.1/3, describing this FAU assay in more details, is available upon request from Novozymes A/S, Denmark, which folder is hereby included by reference.

Acid alpha-amylase activity (AFAU)

Acid alpha-amylase activity is measured in AFAU (Acid Fungal Alpha-amylase Units), which are determined relative to an enzyme standard.

The standard used is AMG 300 L (from Novozymes A/S, glucoamylase wildtype *Aspergillus niger* G1, also disclosed in Boel et al. (1984), EMBO J. 3 (5), p. 1097-1102 and in WO92/00381). The neutral alpha-amylase in this AMG falls after storage at room temperature for 3 weeks from approx. 1 FAU/mL to below 0.05 FAU/mL.

The acid alpha-amylase activity in this AMG standard is determined in accordance with the following description. In this method 1 AFAU is defined as the amount of enzyme, which degrades 5.26 mg starch dry solids per hour under standard conditions.

Iodine forms a blue complex with starch but not with its degradation products. The intensity of colour is therefore directly proportional to the concentration of starch. Amylase activity is determined using reverse colorimetry as a reduction in the concentration of starch under specified analytic conditions.

## Alpha-amylase

Starch + Iodine                      →                      Dextrins + Oligosaccharides

40°C, pH 2.5

Blue/violet                      t=23 sec.                      Decoloration

Standard conditions/reaction conditions: (per minute)

	Substrate:	starch, approx. 0.17 g/L
	Buffer:	Citrate, approx. 0.03 M
	Iodine (I <sub>2</sub> ):	0.03 g/L
5	CaCl <sub>2</sub> :	1.85 mM
	pH:	2.50 - 0.05
	Incubation temperature:	40°C
	Reaction time:	23 seconds
	Wavelength:	lambda=590nm
10	Enzyme concentration:	0.025 AFAU/mL
	Enzyme working range:	0.01-0.04 AFAU/mL

If further details are preferred these can be found in EB-SM-0259.02/01 available on request from Novozymes A/S, and incorporated by reference.

15

Beta-amylase activity (DP°)

The activity of SPEZYME® BBA 1500 is expressed in Degree of Diastatic Power (DP°). It is the amount of enzyme contained in 0.1 ml of a 5% solution of the sample enzyme preparation that will produce sufficient reducing sugars to reduce 5 ml of Fehling's solution when the sample is incubated with 100 ml of substrate for 1 hour at 20°C.

20

Pullulanase activity (New Pullulanase Unit Novo (NPUN))

Pullulanase activity may be determined relative to a pullulan substrate. Pullulan is a linear D-glucose polymer consisting essentially of maltotriosyl units joined by 1,6-alpha-links. Endo-pullulanases hydrolyze the 1,6-alpha-links at random, releasing maltotriose, 6<sup>3</sup>-alpha-maltotriosyl-maltotriose, 6<sup>3</sup>-alpha-(6<sup>3</sup>-alpha-maltotriosyl-maltotriosyl)-maltotriose.

25

One new Pullulanase Unit Novo (NPUN) is a unit of endo-pullulanase activity and is measured relative to a Novozymes A/S Promozyme D standard. Standard conditions are 30 minutes reaction time at 40°C and pH 4.5; and with 0.7% pullulan as substrate. The amount of red substrate degradation product is measured spectrophotometrically at 510 nm and is proportional to the endo-pullulanase activity in the sample. A folder (EB-SM.0420.02/01) describing this analytical method in more detail is available upon request to Novozymes A/S, Denmark, which folder is hereby included by reference.

30

Under the standard conditions one NPUN is approximately equal to the amount of enzyme which liberates reducing carbohydrate with a reducing power equivalent to 2.86 micromole glucose per minute.

35

Determination of CGTase hydrolysis activity

The CGTase hydrolysis activity was determined by measuring the increase in reducing power during incubation with Paselli SA2 starch (from Avebe, The Netherlands) as described by Wind et al. 1995 in Appl. Environ. Microbiol. 61: 1257-1265.

5

Determination of sugar profile and solubilised dry solids

The sugar composition of the starch hydrolysates was determined by HPLC and glucose yield was subsequently calculated as DX. °BRIX, solubilised (soluble) dry solids of the starch hydrolysates were determined by refractive index measurement.

10 **Materials**

The following enzyme activities were used. A maltogenic alpha-amylase with the amino acid sequence shown in SEQ ID No: 1 in WO9/943794. A glucoamylase derived from *Aspergillus oryzae* having the amino acid sequence shown in WO00/04136 as SEQ ID No: 2 or one of the disclosed variants. An acid fungal alpha-amylase derived from *Aspergillus niger*.  
 15 A *Bacillus* alpha-amylase which is a recombinant *B.stearothermophilus* variant with the mutations: I181\*+ G182\*+N193F. A fungal alpha-amylase derived from *Aspergillus oryzae*. A CGTase N with the sequence shown herein as SEQ ID NO 1. A CGTase O with the sequence shown herein as SEQ ID NO 2. A CGTase T with the amino acid sequence disclosed in figure 1 in Joergensen et al (1997) in Biotechnol. Lett. 19:1027-1031 and shown herein as SEQ ID  
 20 NO 3. A CGTase A having the sequence shown herein as SEQ ID NO 4.

Common corn starch (C x PHARM 03406) was obtained from Cerestar.

**Example 1**

This example illustrates the conversion of granular starch into glucose using CGTase  
 25 T and a glucoamylase and an acid fungal amylase. A slurry with 33% dry solids (DS) granular starch was prepared by adding 247.5 g of common corn starch under stirring to 502.5 ml of water. The pH was adjusted with HCl to 4.5. The granular starch slurry was distributed to 100 ml blue cap flasks with 75 g in each flask. The flasks were incubated with magnetic stirring in a 60°C water bath. At zero hours the enzyme activities given in table 1 were dosed to the  
 30 flasks. Samples were withdrawn after 24, 48, 72, and 96 hours.

Table 1. The enzyme activity levels used were:

CGTase T KNU/kg DS	Glucoamylase AGU/kg DS	Acid fungal alpha-amylase AFAU/kg DS
12.5	200	50
25.0	200	50
100.0	200	50

Total dry solids starch was determined using the following method. The starch was completely hydrolyzed by adding an excess amount of alpha-amylase (300 KNU/Kg dry solids) and subsequently placing the sample in an oil bath at 95 °C for 45 minutes. After filtration through a 0.22 microM filter the dry solids was measured by refractive index measurement.

Soluble dry solids in the starch hydrolysate were determined on samples after filtering through a 0.22 microM filter. Soluble dry solids were determined by refractive index measurement and the sugar profile was determined by HPLC. The amount of glucose was calculated as DX. The results are shown in table 2 and 3.

Table 2. Soluble dry solids as percentage of total dry substance at the three CGTase activity levels.

KNU/kg DS	24 hours	48 hours	72 hours	96 hours
12.5	68	82	89	94
25.0	76	89	93	97
100.0	83	96	98	99

Table 3. The DX of the soluble hydrolysate at the three CGTase activity levels.

KNU/kg DS	24 hours	48 hours	72 hours	96 hours
12.5	92.6	94.5	95.1	95.3
25.0	92.4	94.8	95.4	95.5
100.0	92.7	94.9	95.4	95.4

## Example 2

This example illustrates the conversion of granular starch into glucose using CGTase T, a glucoamylase, an acid fungal alpha-amylase and a Bacillus alpha-amylase.

Flasks with 33% DS granular starch were prepared and incubated as described in example 1. At zero hours the enzymes activities given in table 4 were dosed to the flask.

Table 4. The enzyme activity levels used were:

<b>CGTase T KNU/kg DS</b>	<b>Glucoamylase AGU/kg DS</b>	<b>Acid fungal alpha-amylase AFAU/kg DS</b>	<b><i>Bacillus</i> alpha-amylase KNU/kg DS</b>
5.0	200	50	300

Samples were withdrawn after 24, 48, 72, and 96 hours and analyzed as described in example 1. The results are shown in table 4 and 5.

Table 5. Soluble dry solids as percentage of total dry substance.

<b>24 hours</b>	<b>48 hours</b>	<b>72 hours</b>	<b>96 hours</b>
82.8	93.0	96.3	98.7

5

Table 6. The DX of the soluble hydrolysate.

<b>24 hours</b>	<b>48 hours</b>	<b>72 hours</b>	<b>96 hours</b>
92.8	94.9	95.5	95.8

### Example 3

This example illustrates the conversion of granular starch into glucose using a maltogenic alpha-amylase, a glucoamylase and an acid fungal alpha-amylase.

10 Flasks with 33% DS granular starch were prepared and incubated as described in example 1. At zero hours the enzyme activities given in table 6 were dosed to the flasks.

Table 6. The enzyme activity levels used were:

	<b>Maltogenic alpha-amylase MANU/kg DS</b>	<b>Glucoamylase AGU/kg DS</b>	<b>Acid fungal alpha-amylase AFAU/kg DS</b>
Flask 1	5000	200	50
Flask 2	20000	200	50

15 Samples were withdrawn after 24, 48, 72, and 96 hours and analyzed as described in example 1. The results are shown in table 7 and 8.

Table 7. Soluble dry solids as percentage of total dry substance at the two maltogenic alpha-amylase activity levels.

MANU/kg DS	24 hours	48 hours	72 hours	96 hours
5000	63.1	75	79.3	85.3
20000	67.0	77.9	82.7	88.1

Table 8. The DX of the soluble hydrolysate at the two maltogenic alpha-amylase activity levels.

MANU/kg DS	24 hours	48 hours	72 hours	96 hours
5000	95.2	95.4	95.3	95.5
20000	93.8	94.9	94.9	94.8

**Example 4**

This example illustrates the only partial conversion of granular starch into glucose using a glucoamylase and an acid fungal alpha-amylase.

Flasks with 33% DS granular starch were prepared and incubated as described in example 1. At zero hours the enzyme activities given in table 9 were dosed to the flasks. Samples were withdrawn after 24, 48, 72, and 96 hours. The samples were analyzed as described in examples 1. The results are shown in table 10 and 11.

Table 9. The enzyme activity level used were:

Glucoamylase AGU/kg DS	Acid fungal alpha-amylase AFAU/kg DS
200	50

Table 10. Soluble dry solids as percentage of total dry substance.

24 hours	48 hours	72 hours	96 hours
28.5	36.3	41.6	45.7

Table 11. DX of the soluble hydrolysate.

24 hours	48 hours	72 hours	96 hours
27.7	34.9	39.2	42.2

### Example 5

This example illustrates the correlation between the hydrolysis activity of four different CGTases (CGTase A, CGTase N, CGTase O and CGTase T) versus the yield during conversion of granular starch into glucose syrup using a CGTase and a glucoamylase measured as soluble dry solids and development in DX.

Flasks with 33% DS granular starch were prepared and incubated as described in example 1. At zero hour the CGTases were all dosed at 100 KNU/kg DS in combination with glucoamylase at 200 AGU/kg DS. Samples were withdrawn at 48 hours and analyzed as described in examples 1. Results are presented in table 12.

Table 12. Hydrolysis activity (micro mol per min/mg protein), and soluble dry solids (DS) and DX after 48 hours

CGTase	Hydrolysis act.	Soluble DS	DX
CGTase N	0.27	37.4	35.1
CGTase A	0.38	49.9	46.7
CGTase O	1.62	60.9	57.1
CGTase T	4.59	97.9	91.2

### Example 6

This example illustrates the process conducted in an ultrafiltration system where the retentate was held under recirculation in presence of enzymes, raw starch and water and where the permeate is the soluble starch hydrolysate. A slurry comprising 100 kg granular corn starch suspended in 233 L tap city water and CGTase T (12.5 KNU/kg starch), *Bacillus* alpha-amylase (300 KNU/kg starch) and glucoamylase (200 AGU/kg starch) was processed in a batch ultrafiltration system (type PCI) with a tubular membrane module (type PU 120). The slurry was stirred at 100 rpm, pH was adjusted to 4.5 using 170 mL of 30 % HCl, and the reaction temperature was set at 57°C.

Samples of permeate and retentate were analyzed for dry solids content and for sugar composition.

The correction factor for non soluble material is:  $q = (100 - S\%)/(100 - °BRIX)$ . The centrifugation index for sugar is:  $ciS\% = °BRIX/S\%$  (no correction). The theoretical yield of sugar (glucose)  $S_{yield} = ciS\% * q * 100/111 * 100\%$ . A correction has thus been done for 100 kg starch dry matter giving ca. 111 kg glucose dry matter as a result of the hydrolysis reaction.

A trial was made in a simple batch system using the same enzyme system as for the membrane trial. As the comparison in table 15 a and b shows the membrane system reached the maximal solubilisation of starch earlier.

5

Table 13. Dry solids content and sugar composition of retentate and permeate

Sample	Hours	Reactor volume, L	% DS	% DP1	% DP2	% DP3	% DP4
Reactor	3	207	16.1	75.3	10.3	2.6	11.5
Reactor	28	123	28.3	95.0	2.7	0.8	1.5
Reactor	53	123	31.4	95.2	3.4	0.5	0.9
Permeate	3	207	12.1	71.2	17.4	2.9	8.5
Permeate	28	123	21.8	94.9	2.9	0.8	1.3

Tabel 14. Dry solids distribution in retentate at 3, 28, 53 and 77 hours.

	3 hours	28 hours	53 hours	77 hours
Soluble DS	16	28	31	39
Total DS	38	37	42	45

10

Table 15 a. Theoretical yield of glucose versus time for the membrane system

Hours	% total DS in the reactor	°BRIX	$q = (100 - S\%) / (100 - °Brix)$	$cis\% = °Brix / S\%$	Theoretical yield $sis = cis * q * 100 / 111\%$
0	27.0	2.2	0.75	0.08	5
24	35.9	27.3	0.88	0.76	73
48	41.2	30.0	0.84	0.73	89
72	41.2	33.1	0.88	0.80	98
94	41.2	34.8	0.90	0.85	103

15 Table 15 b. Theoretical yield of glucose versus time for a batch reactor system.

Hours	% total DS in the reactor	°BRIX	$q = (100 - S\%) / (100 - °Brix)$	$cis\% = °Brix / S\%$	Theoretical yield $sis = cis * q * 100 / 111\%$
0	29.7	2.0	0.72	0.07	4.
24	29.7	25.6	0.95	0.86	74
48	29.7	28.8	0.99	0.97	86
72	29.7	29.8	1.00	1.00	91
94	29.7	29.8	1.00	1.00	91

The conclusion was that when substrate saturation was maintained during the saccharification in a membrane system the degree of solubilization was improved compared to a simple batch reactor system for cold saccharification of raw starch.

#### Example 7

5 This example illustrates a simultaneous cold liquefaction and saccharification process of the invention carried out in a continuous working microfiltration membrane reactor using a ceramic module.

A 200 L feed mixer tank was connected by a reactor feed pump to a 200 L reactor tank with temperature control. Using a pump with a capacity of 0-20 l/h the mixture from the  
10 reactor was recycled through a APV ceramic microfiltration module for separation of glucose. Pore size was 0.2 micro m and the membrane area was 0.2 m<sup>2</sup>.

The reactor worked for about 200 hours using a dosage of 100 KNU/kg DS CGT-ase T and 300 AGU/kg DS of glucoamylase. With an average holding time in the reactor of 35-45 hours the system operated at steady state for the full period producing a DP1= 93 % glucose  
15 syrup at a yield of close to 100 %.

The reactor tank was loaded with 60 kg of corn starch type Cerestar C x PHARM 03406 suspended in 140 L of tap city water of 58°C under stirring. Using the steam heated mantel the temperature was adjusted to 60°C. Using 30 % HCl the pH was lowered from 6.1 to 4.5. The pH was re-checked (pH=4.5) after 15 minutes. At zero hours, immediately  
20 before adding the enzymes, CGTase T (100 KNU/kg starch) and glucoamylase (300 AGU/kg starch), samples were taken for determination of % sludge volume after centrifugation at 3000 rpm for 3 min in a table centrifuge. Furthermore the °BRIX of the supernatant was measured using a refractometer. The course of the reaction was followed regularly by measurements of sludge volumes and °BRIX of the supernatants as described above.

25 The feed mixer tank was loaded with 186 L of cold tap city water and 80 kg corn starch type Cerestar C x PHARM 03406. The feed mixer was kept stirred gentle and pH was adjusted to 4.5 using 30 % HCl. The temperature was kept at 7-8°C using cooling water and the enzymes CGTase T (100 KNU/kg starch) and glucoamylase (300 AGU/kg starch) was added. The low temperature secured that no reaction took place.

30 The upstart of the reactor was continued until the °Brix-value after 30 hours had stabilized around 27. Then the microfiltration was initiated using a pressure drop of 0.15 Bar and maximal retentate flow to secure this pressure. The filtrate was recycled to the reactor tank the first 5.7 hours. Hereafter the filtrate was collected in a separate tank, and the volume was measured as a function of time. At this point of time the reactor feed pump was started  
35 and adjusted to a flow rate equivalent to the filtrate flux (L/min). By doing so the volume in the reactor tank was kept constant.

The feed of starch slurry was continued while samples were taken as described above. Furthermore samples of the filtrates were taken. Any decrease in the filtrate flux were compensated for by increasing the retentate flow whereby the filter cake on the membrane was disrupted. Thereby the pressure drop was increased too. Samples were taken as a function of time of the filtrate for HPLC and °BRIX as well as the volume collected was measured. Simultaneously samples were taken from the reactor for measuring of total DS, sludge, °Brix and HPLC for sugar composition.

The trial lasted 220 hours. At that point of time the pressure drop was increased to about 0.4 Bar.

Determination of filtrate flux (based on single determinations) and average filtrate flux values (integrated) as a function of the process time showed that the enzyme system consisting of a CGTase and a glucoamylase alone maintained and secured a stable flux over a long processing time. This underlines the industrial potential advantages of this stable system.

The results and a mass balance are presented in tables 16-18.

Table 16. Analyses of collected filtrates.

Date and time	Hours from start	Collected filtrate, L	% DS w/w	Density, kg/L	Mass of DS, kg	Average flux, mL/min
13/03/02 16:05	30*	-	-	-	-	-
14/03/02 16:50	55	142	25.8	1.12	41.1	95.6
16/03/02 16:00	102	187	25.6	1.12	53.7	66.1
18/03/02 13:02	147	200	28.7	1.14	65.2	74.0
19/03/02 16:45	174	100	29.6	1.14	33.8	60.1
Total collected		629.0	27.3	1.13	193.7	-

\*Start of continuous feeding to the reactor

Table 17. Composition of the syrup produced

% DP1	%DP2	% DP3	%DP4
93	5	1	2

Table 18. Mass balance for the trial of example 7

	Mass, kg	% DS	Mass of DS, kg	% Yield of DS*
<b>Upstart of reactor</b>				
Starch	60	90	54	25
Water	140	0	0	
Reactor start	200	27.0	54	25
<b>Continuous production</b>				
Starch consumption (t=28.75 h to t=174.5 h)	235.48	90	212	100
Water consumption (t=28.75 h to t=174.5 h)	548.12	0	0	
Substrate consumption	783.6	27.0	212	100
Syrup production	629.0	27.3	172	81
<b>Reactor at end</b>				
Total content	200	35	70	33
Unconverted starch	18	50	9	4
Mud, L	18	50	9	4
Glucose syrup	164	30	49	23

\*basis substrate consumption at continuous production.

Compared to a batch trial carried out in a simple tank with stirring a significant reduction of the reaction time was obtained using the setup for hydrolysis of granular starch described above. As no viscosity problems were encountered with 30% DS it is considered feasible to increase the DS to 40%, or even as high as 45% and still maintain a smooth operation.

### Example 8

This example compares a process of the invention and a conventional process for production of fuel ethanol or potable alcohol from raw starch in the form of dry milled corn, Yellow Dent No. 2.

A slurry of 30 % DS of dry milled corn was prepared in tap water in 250 ml blue cap flasks and the raw corn starch exposed to simultaneous cold liquefaction and pre-saccharification by a process of the invention. The slurry was heated to 60 °C in a water bath under magnet stirring, pH adjusted to 4.5 using 30 % HCl and CGTase T (75 KNU/kg DS) and glucoamylase (500 AGU/kg DS) added. After 48 hours the flask was cooled in the water bath to 32 °C.

A slurry of 30 % DS dry milled corn was pre-liquefied in a conventional continuous process consisting of a pre-liquefaction vessel, a jet-cooker, a flash, and a post liquefaction vessel. *Bacillus* alpha-amylase was added during the pre-liquefaction at 70-90°C (10 KNU/kg DS) and again during the post liquefaction at ca. 85-90°C (20 KNU/kg DS). The jet-cooking was carried out at 115-120°C. Pre-saccharification was performed under magnet stirring by

heating the mash in blue cap flasks to 60 °C in a water bath. After pH adjustment to 4.5 using 30 % HCl glucoamylase was added in a dosage equivalent to 500 AGU/kg DS. After 48 hours the flask was cooled in the water bath to 32 °C.

- 5 Fermentations were made directly in the blue cap flasks fitted with yeast locks filled with soybean oil. Bakers yeast (*Saccharomyces cerevisiae*) was added in an amount equivalent to 10 millions/mL of viable yeast cells and yeast nutrition in the form of 0.25 % urea was added to each flask. Each treatment was performed in 3 replicates.

- 10 The fermentation was monitored by the CO<sub>2</sub> loss as determined by weighing the flasks at regular intervals. L EtOH/100 kg grain dry matter (DS) was then calculated using the following formula:

$$\text{L EtOH/100 kg mash dry matter} = \frac{\text{Weight loss (g)} \times 1.045}{0.79 \text{ (g/mL)} \times 250 \times 30\% \text{ dry matter}} \times 100$$

The mash contained 30 % w/w grain dry matter. 0.79 g/mL is the density of ethanol.

- 15 Tables 19 and 20 shows the obtained fermentation results for the replicates including the results of statistical calculation of the two types of pretreated raw materials (missing results estimated by interpolation).

Table 19. Fermentation result for the process of the invention using CGTase T (75 KNU/kg DS) and glucoamylase (500 AGU/kg DS).

Hour	L EtOH/100 kg grain	STDEV
0	-	-
25.5	28,3	0.9
48	35,4	0.6
69	37,1	0.2
79	*37,5	-
97	38,3	0.2

\*Estimated value

Table 20. Fermentation result for a conventional process using *Bacillus* alpha-amylase (10+20 KNU/kg DS) and glucoamylase (500 AGU/kg DS)

Hour	L EtOH/100 kg grain	STDEV
0	-	-
25.5	22,5	1.3
48	33,9	0.7
69	*37,2	-
79	38,8	0.4
97	40,5	0.5

\*Estimated value

Using a simulated industrial fermentation time in the interval of approximately 48-70 hours an equivalent or higher alcohol yield was obtained from the mash produced by the process of the invention than could be obtained from a mash produced by the more energy consuming two step hot slurry pre-liquefying and jet-cooking process.

## 5 Example 9

This example illustrates the conversion of granular wheat and common corn starch into glucose using a CGTase, a glucoamylase and an acid fungal alpha-amylase at 60°C.

Flasks with either 33% DS common corn or wheat granular starch were prepared and incubated as described in example 1. At zero hours the enzyme activities given in table 20 were dosed to the flasks. Samples were withdrawn after 24, 48, 72, and 96 hours and analyzed as described in example 1. The results are shown in table 21 and table 22.

Table 20. The enzyme activity levels used were:

CGTase	Glucoamylase	Acid fungal alpha-amylase
NU/g DS	AGU/g DS	AFAU/g DS
100.0	0.2	0.05

Table 21. Soluble dry solids as percentage of total dry substance using two different starch types.

Starch	24 hours	48 hours	72 hours	96 hours
Common corn	85.9	96.2	99.4	100.0
Wheat	95.7	98.9	99.6	100.0

Table 22. The DX of the soluble hydrolysate using the two different starch types.

Starch	24 hours	48 hours	72 hours	96 hours
Common corn	76.2	89.2	93.4	94.7
Wheat	86.2	92.4	93.6	94.4

**CLAIMS**

- 5 1. A one step process for producing a soluble starch hydrolysate, the process comprising  
subjecting a aqueous granular starch slurry at a temperature below the initial  
gelatinization temperature of said granular starch to the simultaneous action of;  
  
a first enzyme which;  
  
(a) is a member of the Glycoside Hydrolase Family13;  
  
(b) has alpha-1.4-glucosidic hydrolysis activity, and;  
10 (c) comprises a Carbohydrate-Binding Module Family 20,  
  
and at least one second enzyme which is a fungal alpha-amylase (EC 3.2.1.1), a  
beta-amylase (E.C. 3.2.1.2), or a glucoamylase (E.C.3.2.1.3).
- 15 2. The process of the preceding claim, wherein the starch slurry has 20-55% dry solids  
granular starch, preferably 25-40% dry solids granular starch, more preferably 30-35%  
dry solids, especially around 33% dry solids granular starch.
3. The process of any of the preceding claims, wherein at least 85%, 86% , 87%, 88%,  
89% least 90%, 91%, 92%, 93% 94%, 95%, 96%, 97%, 98% or at least 99% of the dry  
solids of the granular starch is converted into a soluble starch hydrolysate.
- 20 4. The process of any of the preceding claims, wherein the first enzyme is of microbial  
origin, and preferably of bacterial origin.
5. The process of any of the preceding claims, wherein the first enzyme is a CGTase (EC  
2.4.1.19).
- 25 6. The process of any of the preceding claims, wherein the first enzyme is a CGTase  
having a hydrolysis activity of at least 3.5, preferably at least 4, 4.5, 5, 6, 7, 8, 9,10, 11,  
12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, or most preferably at least 23 micro mol per  
min/mg.
- 30 7. The process of any of the preceding claims, wherein the first enzyme is a CGTase  
having 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or even 90% homology to the  
amino acid sequence shown in figure 1 in Joergensen et al. (1997), Biotechnol. Lett.  
19:1027-1031.

8. The process of any of the preceding claims, wherein the first enzyme is a maltogenic alpha-amylase (E.C. 3.2.1.133).
9. The process of any of the preceding claims, wherein the maltogenic alpha-amylase is derived from *Bacillus*, preferably from *B. stearothermophilus*.
- 5 10. The process of any of the preceding claims wherein the first enzyme is a maltogenic alpha-amylase having 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or even 90% homology to the amino acid sequence shown in SEQ ID NO:1 in WO9943794.
- 10 11. The process of any of the preceding claims, wherein the first enzyme is the maltogenic alpha-amylase having the amino acid sequence shown SEQ ID NO:1 in WO9943794 or a variant of said amino acid sequence disclosed in said patent.
12. The process of any of the preceding claims, wherein the first enzyme is a maltogenic alpha-amylase having a hydrolysis activity of at least 3.5, preferably at least 4, 4.5, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 100, 200, 300, 400, 500, 600, or most preferably at least 700 micro mol per min/mg.
- 15 13. The process of any of the preceding claims, wherein the second enzyme is a fungal alpha-amylase, having 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or even 90% homology to the amino acid sequence shown in SEQ ID NO:10 in WO9623874.
14. The process of any of the preceding claims, wherein the second enzyme is a barley beta-amylase (E.C. 2.4.1.2), such as Spezyme® BBA 1500 or Spezyme® DBA from Genencor int.
- 20 15. The process of any of the preceding claims, wherein the second enzyme is a glucoamylase.
16. The process of any of the preceding claims, wherein the second enzyme is a glucoamylase derived from *Aspergillus oryzae*, such as a glucoamylase having 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or even 90% homology to the amino acid sequence shown in SEQ ID NO:2 in WO00/04136.
- 25 17. The process of any of the preceding claims wherein a third enzyme is present, said third enzyme being an alpha-amylase derived from a *Bacillus* sp., such as the enzymes, the variants and hybrids disclosed in WO99/19467, WO96/23874, WO97/41213, and WO99/19467.
- 30 18. The process of any of the preceding claims, wherein a third enzyme is present, said enzyme being an isoamylase or a pullulanase.

19. The process of any of the preceding claims, wherein the temperature is at least 58°C, 59°C, or more preferably at least 60°C.
20. The process of any of the preceding claims, wherein the pH is in the range of 3.0 to 7.0, preferably from 3.5 to 6.0, or more preferably from 4.0-5.0.
- 5 21. The process of any of the preceding claims, wherein the soluble starch hydrolysate has a DX of at least 94.5%, 95.0%, 95.5%, 96.0%, 96.5%, 97.0%, 97.5%, 98.0%, 98.5, 99.0% or at least 99.5%.
22. The process of any of the preceding claims, wherein the dominating saccharide in the soluble starch hydrolysate is glucose or maltose.
- 10 23. The process of any of the preceding claims, wherein the granular starch is obtained from tubers, roots, stems, or whole grain.
24. The process of any of the preceding claims, wherein the granular starch is obtained from cereals.
- 15 25. The process of any of the preceding claims, wherein the granular starch is obtained from corn, cobs, wheat, barley, rye, milo, sago, cassava, tapioca, sorghum, rice or potatoes.
26. The process of any of the preceding claims, wherein the granular starch is obtained from dry milling of whole grain or from wet milling of whole grain.
- 20 27. The process of any of the preceding claims, wherein the process is conducted in an ultrafiltration system and where the retentate is held under recirculation in presence of enzymes, raw starch and water and where the permeate is the soluble starch hydrolysate.
- 25 28. The process of any of the preceding claims, wherein the process is conducted in a continuous membrane reactor with ultrafiltration membranes and where the retentate is held under recirculation in presence of enzymes, raw starch and water and where the permeate is the soluble starch hydrolysate.
- 30 29. The process of any of the preceding claims, wherein the process is conducted in a continuous membrane reactor with microfiltration membranes and where the retentate is held under recirculation in presence of enzymes, raw starch and water and where the permeate is the soluble starch hydrolysate.

30. A process for production of high fructose starch-based syrup (HFSS), wherein a soluble starch hydrolysate of the process of any of the preceding claims is subjected to conversion into high fructose starch-based syrup (HFSS), such as high fructose corn syrup (HFCS).
- 5 31. A process for production of fuel or potable ethanol, wherein a soluble starch hydrolysate of the process of any of claims 1-29 is subjected to fermentation into ethanol.
32. The process of claim 31, wherein the fermentation step is carried out simultaneously or separately/sequential to the hydrolysis of the granular starch.
- 10 33. The process of any of the claims 31-32, wherein the process is conducted in an ultrafiltration system where the retentate is held under recirculation in presence of enzymes, raw starch, yeast, yeast nutrients and water and where the permeate is an ethanol containing liquid.
- 15 34. The process of any of the claims 31-33, wherein the process is conducted in a continuous membrane reactor with ultrafiltration membranes and where the retentate is held under recirculation in presence of enzymes, raw starch, yeast, yeast nutrients and water and where the permeate is an ethanol containing liquid.

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# United States Patent [19]

Kainuma et al.

[11] Patent Number: 4,591,560

[45] Date of Patent: May 27, 1986

[54] PROCESS FOR SACCHARIFICATION OF STARCH USING ENZYME PRODUCED BY FUNGUS BELONGING TO GENUS CHALARA

[75] Inventors: Keiji Kainuma; Shoichi Kobayashi, both of Sakura, Japan

[73] Assignee: Director of National Food Research Institute Ministry of Agriculture, Forestry and Fisheries, Yatabe, Japan

[21] Appl. No.: 566,499

[22] Filed: Dec. 29, 1983

[30] Foreign Application Priority Data

Jan. 17, 1983 [JP] Japan ..... 58-5564

[51] Int. Cl.<sup>4</sup> ..... C12P 19/20; C12P 19/14; C12N 9/30; C12N 9/34; C12R 1/645

[52] U.S. Cl. .... 435/96; 435/99; 435/203; 435/205; 435/911

[58] Field of Search ..... 435/99, 96, 201, 202, 435/203, 911, 254, 255, 205

## [56] References Cited

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Primary Examiner—Thomas G. Wiseman

Assistant Examiner—Jayme A. Huleatt

Attorney, Agent, or Firm—Frishauf, Holtz, Goodman & Woodward

## [57] ABSTRACT

A process for the saccharification of starch, which comprises saccharifying a raw and/or gelatinized starch by the use of an amylase produced by a fungus belonging to genus Chalara to produce glucose.

According to the process of the present invention, the starch is directly saccharified, and glucose can be obtained efficiently.

10 Claims, 5 Drawing Figures

FIG. 1

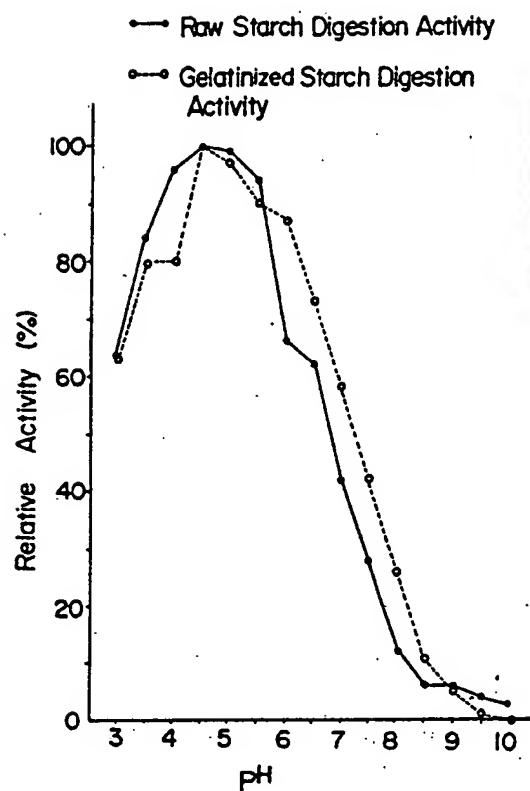


FIG. 2

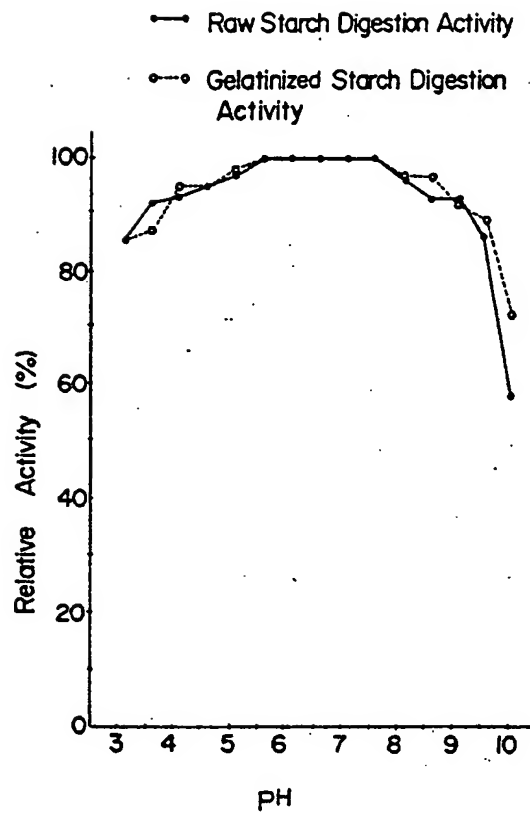


FIG. 3

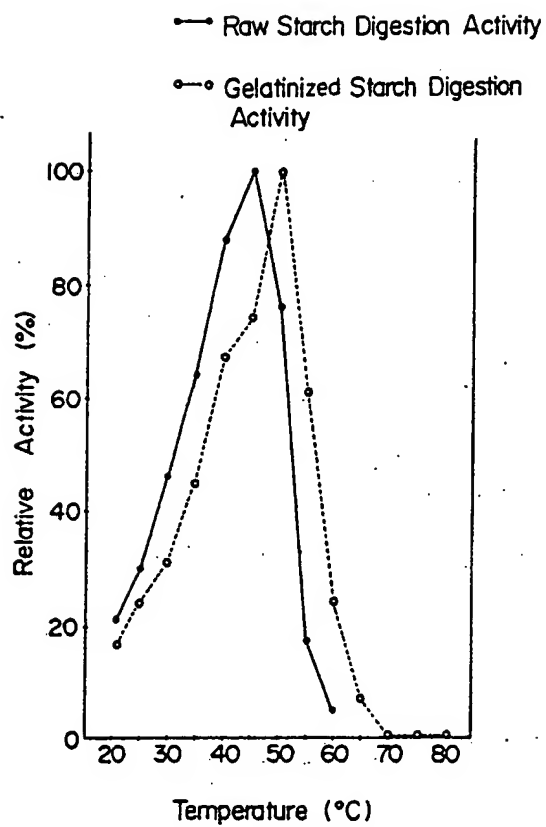


FIG. 4

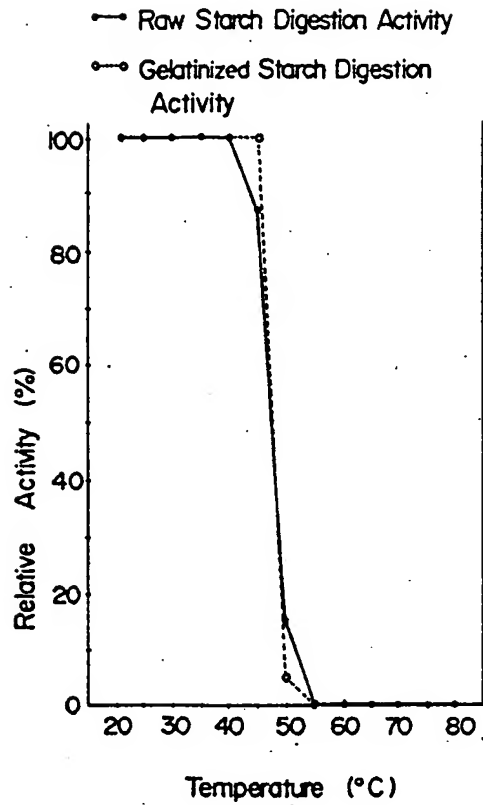
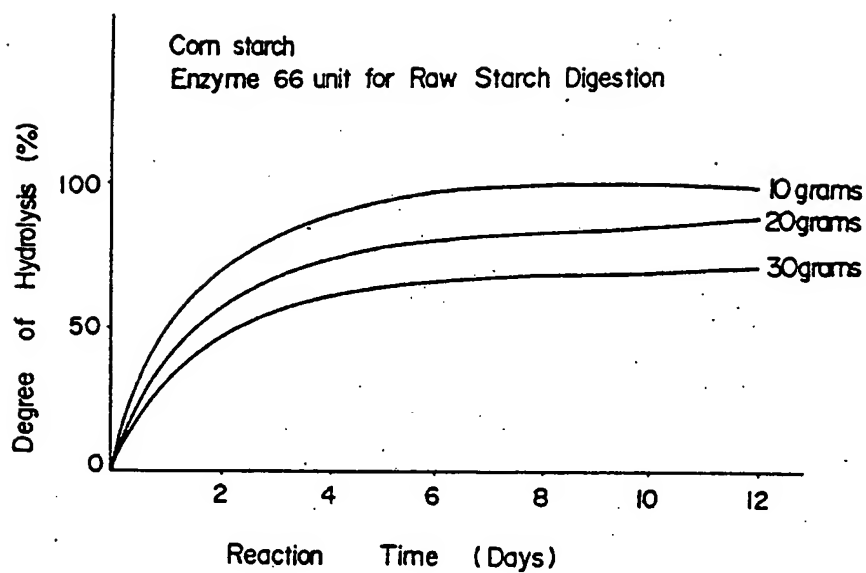


FIG. 5



# PROCESS FOR SACCHARIFICATION OF STARCH USING ENZYME PRODUCED BY FUNGUS BELONGING TO GENUS CHALARA

## BACKGROUND OF THE INVENTION

In the usual saccharification of starch, starch is once cooked to gelatinize, this gelatinized starch is liquefied by the action of  $\alpha$ -amylase and, thereafter, glucoamylase is added to produce dextrose. This method, however, requires a large amount of energy for gelatinizing the starch prior to its saccharification. In order to minimize such energy consumption, extensive researches have been made particularly in recent years for amylase which can be applied directly to raw starch, that is, is capable of hydrolyzing directly the raw starch.

Such energy-saving starch saccharification is essential for the production of alcohol fuel from various starches as biomass sources. In connection with the alcoholic fermentation of raw starch, investigations by S. Ueda et al., Hayashida et al., Y. K. Park et al., and so on are known. S. Ueda et al. have long studied the alcoholic fermentation of raw starch using glucoamylase produced by *Black Aspergillus*, *Asp. awamori* (see S. Ueda & Y. Koba, *J. Fermentation Technology*, 58, No. 3, 237 (1980), and S. Ueda et al., *Biotech. Bioeng.*, Vol. 23, 291 (1981)). Hayashida et al. report that amylase produced by *Asp. awamori* is more effective in the hydrolysis of raw starch than amylase produced by *Asp. oryzae* or malt amylase. Y. K. Park et al. report the studies on the alcohol fermentation of starch without gelatinizing starch, using glucoamylase produced by *Aspergillus niger* or *Aspergillus awamori* (see *Biotech. Bioeng.*, 24, 495 (1982)).

In the saccharification of raw starch using glucoamylases produced by *Aspergillus niger*, *Aspergillus awamori*, or fungus belonging to genus *Rhizopus*, some problems still remain unsolved. The most serious problem is that the rate of hydrolysis of raw starch of the above-described enzymes are seriously low compared with their rate of hydrolysis of gelatinized starch. In other words, their raw starch-hydrolyzing activity is seriously low although they have high enzymatic activity. Usually it is considered that enzymes capable of hydrolyzing raw starch at a rate of hydrolysis of about 1/30 of that for gelatinized starch are promising as raw starch-hydrolyzing enzymes (see S. Ueda, Workshop, *Carbohydrate Sources and Biotechnology*, page 25 (1982), held under the auspices of National Food Research Institute, Japan and sponsored by The United Nations University).

About 2,000 strains of microorganisms living in soil and on wood were isolated by us and examined to find those microorganisms satisfying the requirement that the ratio of the gelatinized starch-hydrolyzing degree to the raw starch-hydrolyzing degree is 10:1 or less. It has been found that some microorganisms satisfy the foregoing requirement. They are strains belonging to genus *Chalara*, and the properties of the enzymes secreted by them are similar to those of glucoamylases in respect of the mechanisms of enzyme reactions. These enzymes are active and stable in a slightly acidic region and have greatly higher raw starch-hydrolyzing activity compared with conventional glucoamylases; that is, the ratio of the gelatinized starch-hydrolyzing activity to the raw starch-hydrolyzing activity is from 3.5:1 to 5:1, which is greatly higher compared with those of the known glucoamylases. Hence it has been found that the

saccharification of raw starch can be performed advantageously on a commercial scale by using the above-described enzymes.

## SUMMARY OF THE INVENTION

The present invention relates to a process for the saccharification of starch (raw or gelatinized), characterized by saccharifying a starch by the use of an amylase produced by a fungus belonging to genus *Chalara* to produce dextrose directly.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a curve showing activities at different pH values of a crude enzyme produced by *Chalara paradoxa* PNS-80 of the invention, said activity being indicated as relative value of raw starch-hydrolyzing activity to gelatinized starch-hydrolyzing activity; said activities are determined by incubating said enzyme with corn starch (raw starch) at 30° C. for 30 minutes or by incubating said enzyme with soluble starch (gelatinized starch) at 40° C. for 30 minutes. It discloses that activity is present over the range of pH of from about 3.5 to 9.5 and good results are obtained up to a pH of about 7, with best results obtained from about 3.5 to 6.

FIG. 2 is a pH stability curve (over the range of 3-9.5) as determined by measuring the residual activity of an enzyme solution after its treatment at 40° C. for 30 minutes, provided that the measurement conditions are the same as in FIG. 1.

FIG. 3 is a curve showing the optimum temperature of the present enzyme produced by *Chalara paradoxa* PNS-80, which is obtained by plotting relative enzyme activities as determined by incubating said enzyme with starch at a predetermined temperature for 30 minutes. It further discloses a broad temperature range when using raw starch of about 30° to 50° C. and that best results for raw starch are obtained at temperatures of about 40° to 48° C.

FIG. 4 shows temperature stability of the present enzyme produced by *Chalara paradoxa* PNS-80, and it is obtained by plotting the residual activity of an enzyme solution after its treatment at a predetermined temperature for 30 minutes.

FIG. 5 is a raw corn starch-hydrolyzing curve as determined under the conditions described in Example 7.

## DETAILED DESCRIPTION OF THE INVENTION

Any fungi belonging to genus *Chalara* and having an ability to produce enzyme of high raw starch-hydrolyzing ability can be used in the invention. A typical example is a strain *Chalara paradoxa* PNS-80. Its microbiological characteristics as determined based on K. Tsubaki and S. Udagawa, *A PICTURE BOOK OF FUNGI* (last volume), Kodansha Life Scientific Publishers are as follows:

### (I) Morphological Characteristics

(a) It forms conidiophores growing vertically from hyphae, which have a long cylindrical form and 2 to 3 septa.

(b) It forms phialo type conidia which are cylindrical or barrel-like in shape and have an average size of 12 microns  $\times$  4 microns.

(c) It forms brown or gray-black thick wall spores, the skin layer of which is covered with a smooth or irregular external wall.

## (II) Culture Characteristics

(a) It grows on a malt agar medium in the form of gray or gray-yellow flocculence. At a later stage of the growth, it further becomes dark.

(b) It grows on a medium comprising 1% soluble starch, 1% polypeptone, and 0.7% bouillon in the same flocculent form as in (a) above.

In view of the above-described morphological and culture characteristics, it is reasonable to identify the strain as *Chalara paradoxa*. This strain has been deposited in the Fermentation Research Institute under the accession number of FERM BP-422.

The desired enzymes can be prepared by cultivating the fungus belonging to genus *Chalara* by the usual aerobic liquid cultivation or cultivation aerated with agitation. For this purpose, various culture media can be used. As a carbon source, raw starch from corn, potato, sweet potato, tapioca, waxy corn, rice, wheat, sago, high-amylose corn and so forth, and a starch hydrolyzate of DE 10-25 are preferred to use in a proportion of from 2 to 7%, since the desired enzymes are induced using raw starch. As a nitrogen source, peptone, meat extract, corn steep liquor, peptide-containing compounds, and so forth can be used singly or in combination with each other. If desired, small amounts of inorganic salts, such as NaCl, FeSO<sub>4</sub>, Ba(OH)<sub>2</sub>, FeCl<sub>3</sub>·6H<sub>2</sub>O, SrCl<sub>2</sub>·6H<sub>2</sub>O, LiCl, MgSO<sub>4</sub>·7H<sub>2</sub>O, and MnSO<sub>4</sub>·5H<sub>2</sub>O, can be added.

The thus-prepared culture medium is inoculated with the above-described strain, which is then cultivated under aerobic conditions at pH 4-8.5 at 25°-40° C. for 24-96 hours, whereby the desired enzyme can be accumulated therein.

The enzyme used in the present process may be utilized when still part of the culture broth obtained by cultivating the amylase-producing microorganism belonging to genus *Chalara* on a nutrient medium in the manner described above, in the form of its filtrate, in the form of a concentrated filtrate, and in the form of a purified enzyme prepared from the filtrate.

Separation and purification of the present enzyme can be performed by the known procedures which have been widely used in the separation and purification of enzymes from their culture broth. For example, a method of concentrating the filtrate under reduced pressure or by ultrafiltration, a method of salting out with compounds such as ammonium sulfate, sodium sulfate and sodium chloride, a specific adsorption method utilizing raw starch, a fractional precipitation method using compounds such as methanol, ethanol and acetone, a chromatographic method using DEAE-Sephadex and an ion exchange resin, an isoelectric point precipitation method, and an electric dialysis method can be used singly or in combination with each other.

The activity of the enzyme is measured as follows:

A mixture of 20 milligrams of raw corn starch, 0.2 milliliter of a 0.1 M acetate buffer (pH: 4.5), 0.2 milliliter of an enzyme solution, and 1.6 milliliters of deionized water is incubated at 40° C. for 30 minutes. At the end of the time, the amount of glucose formed is measured by the Somogyi-Nelson method. One unit of enzyme activity is defined as the amount of enzyme which produces 1 micromole (180 micrograms) of glucose per minute under the conditions as described above.

The gelatinized starch-hydrolyzing activity is determined by measuring the amount of reducing sugar formed when the same experiment as above is performed using 0.25 milliliter of a 2% soluble starch solu-

tion. One unit of the activity is defined in the same manner as above.

The physical and chemical properties of the present enzyme are shown below. This enzyme is the one isolated by ultra filtration of the culture filtrate.

## (1) Action and Substrate Specificity

The present enzyme is capable of hydrolyzing raw and gelatinized starches from corn, potato, rice, sweet potato, waxy corn, sago and tapioca, yielding reducing sugar. Paper chromatography and high-pressure liquid chromatography analyses of the reducing sugar show that oligosaccharides including disaccharide are not formed and glucose is formed and accumulated from the initial stage of the reaction. It is believed, therefore, that the present enzyme is a glucoamylase which converts starch into glucose by an exo-type reaction.

## (2) Optimum pH and Stable pH Range

The optimum pH and stable pH range were determined by applying a 0.2 M acetate buffer (pH: 3-5.0), a trisphosphate buffer (pH: 5.5-8.5), and a sodium carbonate buffer (pH: 9.0-10.0) to raw starch at 40° C. for 30 minutes. The results are shown in FIGS. 1 and 2.

## (3) Optimum Temperature and Temperature Stability

The optimum temperature was determined by measuring the formed reducing sugar after 30 minutes incubation with soluble starch solution, and temperature stability were determined by the remaining enzyme activity after its treatment for 30 minutes at different temperatures. Experimental results obtained using gelatinized soluble starch as a substrate are shown in FIGS. 3 and 4. Since the present enzyme is intended to apply to raw starch, it is used within the stable temperature range thereof.

## (4) Influences of Coexisting Ions

Addition of calcium (Ca) ion increases the thermal stability of the gelatinized starch-hydrolyzing activity by about 5° C.

Glucose can be formed by application of the present enzyme to raw starch and/or gelatinized starch.

Strains capable of producing the present enzyme include, as well as *Chalara paradoxa* PNS-80, *Chalara fusidioides*, *Chalara cylindrosperma*, *Chalara mycoderma*, *Chalara quercina* and *Chalara elegans*.

The present invention permits the direct saccharification of starch and, therefore, it provides a process for producing conveniently and efficiently useful substances such as alcohols from various starches such as biomass sources. In particular, the enzyme of the invention is suitable for industrial utilization because of its high raw starch-hydrolyzing activity.

The following Examples are for illustrative purposes only and are not meant to limit the invention set forth in the claims appended hereto.

## EXAMPLE 1

One liter of a medium containing 65 grams of corn steep liquor, 7 grams of meat extract, 3 grams of sodium chloride, and 500 milligrams of ferrous sulfate (adjusted to pH 4.0) was placed in a 5-liter flask and sterilized. After sterilization, 40 grams of sago starch which had been subjected to dry air sterilization at 100° C. was added to the medium, which was then inoculated with one loop of slant culture of *Chalara paradoxa* PNS-80 (FERM BP-422). Shaking cultivation was performed at 30° C. for 5 days.

After the cultivation was completed, microorganisms and unreacted starch were removed by centrifugal separation, and a supernatant was used as a crude enzyme

solution (raw starch-hydrolyzing activity: 1.0 International Unit (IU) per milliliter).

Granules, i.e. raw, waxy corn starch, corn starch, and wheat starch were placed in the respective Erlenmeyer flasks each in an amount of 2.5 grams. Then 25 milliliters of the enzyme solution as prepared above was added to each flask. Furthermore 25 milliliters of a 0.1 M acetate buffer (pH 4.5) and 200 milliliters of deionized water were added, and shaking cultivation was performed at 30° C.

The amount of glucose formed was measured by the Somogyi-Nelson method. The degree of hydrolysis of each starch after 24 hour reaction was as follows:

Corn starch: 68%

Waxy corn starch: 85.5%

Wheat starch: 76.3%

Paper chromatography analysis shows that the sugar formed consisted of dextrose alone.

#### EXAMPLE 2

The same strain as used in Example 1 was cultivated on a medium consisting of 65 grams of corn steep liquor, 3 grams of sodium chloride, and 70 grams of tapioca which had been sterilized with radiation. After the cultivation was completed, 2 volumes of ethanol was added to one volume of the fermentation broth to precipitate an enzyme. To 50 milligrams of the precipitate were added 50 milliliters of a 0.1 M acetate buffer (pH 4.5) and 200 milliliters of deionized water.

Thereafter rice starch, potato starch, and sweet potato starch were hydrolyzed in the same manner as in Example 1. The degree of hydrolysis of each starch after 24 hour reaction was as follows:

Rice starch: 89%

Potato starch: 11%

Sweet potato starch: 48.6%

#### EXAMPLE 3

One liter of a medium containing 65 grams of corn steep liquor, 7 grams of meat extract, 3 grams of sodium chloride, and 500 milligrams of ferrous sulfate (adjusted to pH 4.0) was placed in a 5-liter Erlenmeyer flask and sterilized. After the completion of sterilization, 70 grams of sago starch which had been sterilized with 7-ray radiation was added to the medium, which was then inoculated with one loop of slant culture of the same strain as used in Example 1. Shaking cultivation was performed with shaking for 6 days.

After the cultivation was completed, the fermentation broth was subjected to centrifugal separation to obtain a supernatant. This supernatant was used as a crude enzyme solution (1.6 International Units per milliliters (IU/ml)) in the raw starch-hydrolyzing reaction as described below.

Rice starch, waxy corn starch, and wheat starch were placed in the respective Erlenmeyer flasks each in an amount of 5 grams. Then 25 milliliters of the enzyme solution as prepared above, 25 milliliters of a 0.1 M acetate buffer (pH 4.5), and 200 milliliters of deionized water were added to each flask. Shaking cultivation was performed at 30° C. for 48 hours.

The amount of glucose formed was measured by the Somogyi-Nelson method. The degree of hydrolysis of each starch was as follows:

Rice starch: 86%

Waxy corn starch: 86%

Wheat starch: 86%.

#### EXAMPLE 4

The procedure of Example 3 was repeated wherein tapioca starch and sweet potato starch were used as the substrate in place of the rice starch, waxy corn starch, and wheat starch.

The degree of hydrolysis of each starch was as follows:

Tapioca starch: 89.8%

Sweet potato starch: 84%

#### EXAMPLE 5

The procedure of Example 3 was repeated wherein sago starch and potato starch were used as the substrate in place of the rice starch, waxy corn starch, and wheat starch.

The degree of hydrolysis of each starch was as follows:

Sago starch: 52%

Potato starch: 35%.

#### EXAMPLE 6

One liter of a medium containing 65 grams of corn steep liquor, 7 grams of meat extract, 3 grams of sodium chloride, and 300 milligrams of ferrous sulfate (adjusted to pH 4.0) and placed in a 5-liter flask and sterilized. After sterilization, 70 grams of sago starch which had been sterilized with radiation was added to the medium, which was then inoculated with one loop of slant culture of the same strain as used in Example 1. Shaking cultivation was performed for 5 days. Microorganisms were removed by centrifugal separation, and a supernatant was used as a crude enzyme solution (activity for gelatinized starch: 14.9 International Units per milliliter (IU/ml); activity for raw starch: 1.6 International Units per milliliter (IU/ml)).

A mixture of 2.5 grams of each of the raw starches as described in the Table, 12.5 milliliters of a 0.1 M acetate buffer (pH 4.0), 100 milliliters of deionized water, and 12.5 milliliters of the crude enzyme solution as prepared above was incubated at 30° C. After 24 hours and 48 hours, the degree of hydrolysis of the starch was measured. The results are shown in the Table below.

TABLE

Starch	Time	
	24 hours	48 hours
Rice starch	95.0	96.1
Waxy corn starch	93.5	97.3
Wheat starch	91.5	96.2
Corn starch	90.9	95.6
Tapioca	81.2	99.8
Sweet potato starch	73.3	92.6
Sago starch	33.5	57.7
Potato starch	19.4	38.6

#### EXAMPLE 7

A mixture of 42 milliliters of an enzyme solution as prepared in Example 6 (raw starch-hydrolyzing activity: 66 International Units in 40 milliliters (IU/ml)), 20 milliliters of a 0.1 M acetate buffer, and 38 milliliters of deionized water was prepared in a 500-milliliter flask. To this enzyme solution was added raw corn starch in an amount of 10 grams, 20 grams or 30 grams. The degree of hydrolysis at each amount was measured and plotted to obtain a hydrolysis curve as shown in FIG. 5. When the amount of the starch added was 10 grams, the degree of hydrolysis after 7 day incubation was 100%;

when the amount of the starch added was 20 grams, the degree of hydrolysis after 9 day incubation was 90%; and when the amount of the starch added was 30 grams, the degree of hydrolysis after 9 day incubation was 72%.

What is claimed is:

1. A process for the saccharification of starch to produce glucose, which comprises contacting a starch with an amylase produced by a fungus belonging to the genus *Chalara* to produce glucose in an aqueous medium having a pH of from 3 to 9.5 at a temperature of from 30° to 50° C. to produce said glucose, and recovering said glucose, said amylase has a pH stability of 3-9.5, an optimum temperature of 45° C. for raw starch and of 50° C. for gelatinized starch and thermostability of 45° C.

2. The process of claim 1, wherein said starch is a raw starch.

3. The process of claim 2, wherein said pH is up to 7 and said temperature is between 40° and 48° C.

4. The process of claim 3, wherein said pH is between about 3.5 and 6.

5. The process of claim 4, wherein said fungus belonging to the genus *Chalara* is *Chalara paradoxa* PNS-80 (FERM BP- 422).

6. The process of claim 1, wherein said fungus belonging to the genus *Chalara* is *Chalara paradoxa* PNS-80 (FERM BP- 422).

7. The process of claim 1, wherein said fungus belonging to the genus *Chalara* is selected from the group consisting of *Chalara quercina* and *Chalara elegans*.

8. The process of claim 1, wherein said aqueous medium also contains calcium ion.

9. A process for producing an amylase which has high activity in digesting starch to form glucose comprising cultivating *Chalara paradoxa* PNS-80 (FERM BP-422) in a culture medium containing a nitrogen source under aerobic conditions at a pH of 4-8.5 and at a temperature of 25°-40° C. to produce said amylase, said amylase has a pH stability of 3-9.5, an optimum temperature of 45° C. for raw starch and of 50° C. for gelatinized starch and thermostability of 45° C.

10. The process of claim 9, wherein said amylase is separated from said culture medium.

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# United States Patent [19]

Dwiggins et al.

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## [54] RAW STARCH SACCHARIFICATION

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[52] U.S. Cl. .... 435/96; 435/99; 435/205; 435/911

[58] Field of Search ..... 435/96, 99, 205

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## [57] ABSTRACT

A multi-step process is provided for solubilizing and saccharifying granular starch slurries of between 20 and 60% d.s. starch. In a first step, an enzyme mixture exhibiting raw starch hydrolyzing activity is added to the slurry and maintained in contact therewith until at least a substantial portion but less than all of the starch is solubilized. The resulting syrup is separated from the remaining insoluble fraction. In a second step, the insoluble fraction is used to prepare a second slurry containing less than about 20% d.s. starch, and this second slurry is solubilized with a raw starch hydrolyzing enzyme preparation until substantially all (e.g., at least about 95% or more) of the starch is solubilized. The pooled syrup from the two steps is over about 20 weight percent saccharides, at least about 95% of which is glucose.

24 Claims, No Drawings

## RAW STARCH SACCHARIFICATION

## FIELD OF THE INVENTION

The present invention is directed to the conversion of starch to glucose, and more particularly to direct enzymatic conversion of a high solids granular starch slurry directly to glucose.

## BACKGROUND OF THE INVENTION

A large number of processes have been described for converting starch to a monosaccharide, glucose. Glucose has value in itself, and also as a precursor for other saccharides such as fructose. Glucose may also be fermented to ethanol or other fermentation products.

The ability of alpha-amylase to hydrolyze raw (un-pasted) starch and produce water soluble oligosaccharides has been known since the early 1900's, Reichert, E. T., *Publication of the Carnegie Institution at Washington*, No. 173, Part 1 (1913). Subsequently, other enzymes have been found that hydrolyze unpasted starch, most notably glucoamylase, which converts granular starch directly to glucose, Evers, A. D. et al., *Die Starke* 23 (1971), p. 16. The ability of an enzyme to hydrolyze granular starch is associated with the enzyme's ability to be adsorbed on the starch, Walker, G. J. et al., *Biochemical Journal* 86 (1963), p. 452. The extent of hydrolysis is related to the specific type of starch (corn, potato, wheat, etc.); and its physical condition (granule size; granules intact, fractured, swollen, abraded, etc.), the specific enzyme type and source; the starch and enzyme concentration; the temperature; the pH; and other factors, (Jones, C. R., *Cereal Chemistry*, 17 (1940), p. 133; Sandstedt, R. M. et al., *Journal of the Japanese Society of Starch Science*, Vol. 17 (1965), p. 215). The particular effects of these variables are generally only empirically understood.

Several processes for the commercial, low temperature, enzymatic solubilization of granular starch have been proposed, U.S. Pat. Nos. 2,583,451 issued Jan. 22, 1952 to Wallerstein et al; 3,922,196 issued Nov. 25, 1975 to Leach et al; 3,922,197 issued Nov. 25, 1975 to Leach et al; 3,922,198 issued Nov. 25, 1975 to Kuske et al; 3,922,199 issued November 1975 to Hebeda et al; 3,922,200 issued Nov. 25, 1975 to Walon et al and 3,922,201 issued Nov. 25, 1975 to Hebeda et al. Generally, an alpha-amylase is used at pH 4 to 6 at 40° C. to 75° C. to produce a low D. E. syrup. Simultaneously or subsequently, a saccharifying enzyme, such as fungal alpha-amylase, beta-amylase, or glucoamylase may be used at a suitable pH and temperature to produce the desired hydrolysis. The major advantage of such procedures are that they eliminate the high temperature cooking step at 100° C. to 150° C. in presently used syrup processes (U.S. Pat. No. 3,783,100 issued Jan. 1, 1974 to R. F. Larson et al and U.S. Pat. No. 3,875,140 issued Apr. 1, 1975 to Barker et al). Important disadvantages are increased enzyme costs, incomplete solubilization of starch resulting in the need for subsequent starch recycle, mud (fiber, protein and fat) separation problems, and microbial contamination problems.

Recently, it has been discovered that certain fungi, particularly species of the genus *Humicola* secrete a mixture of enzymes, including enzymes with glucoamylase activity, which efficiently hydrolyze raw (un-pasted) starch granules. The ability to hydrolyze starch in granular form is referred to herein as raw starch hydrolyzing (RSH) activity. Enzyme preparations hav-

ing, in either crude or more refined forms, RSH activity will be referred to herein as RSH enzyme preparations.

RSH preparation obtained from species of the genus *Humicola*, particularly the species *Humicola grisea* var. *thermoidea*, which is hereinafter referred to as *Humicola RSH enzyme preparation*, has the following characteristics. The *Humicola RSH enzyme preparation* hydrolyzes raw or granular starch, including straight- and branch-chained starches, and hydrolyzes the starch substantially entirely to glucose. This enzyme preparation is characterized by including a glucoamylase enzyme (EC 3.2.1.3) having an isoelectric point higher than pH 8.0 and a proteinaceous material having glucoamylase-potentiating activity which, in cooperation with the glucoamylase, catalyzes the hydrolysis of the granular starch. Said enzyme preparation is further characterized in that the glucoamylase fraction adsorbs on carboxymethyl cellulose, whereas a fraction containing material exhibiting potentiating activity, or "potentiating factor", is not adsorbed by carboxymethyl cellulose. The *Humicola RSH preparation* has the ability, for example, to hydrolyze granular starch in a 15% starch solids suspension in water to a solution of saccharides of at least 97% by weight glucose, dry substance basis (d.s.b.), with essentially no starch residue in the absence of debranching enzyme or added alpha-amylase when the hydrolysis is carried out at a pH of between about 5.0 and 7.0, the optimum pH range, and at a temperature of 55° C.

Although certain RSH enzyme preparations will substantially completely hydrolyze granular starch slurries at about 15 weight percent starch, dry solids (d.s.) to produce a high percentage glucose syrup, they generally do not completely solubilize starch in slurries of higher concentration within a reasonable time or provide sufficiently high percentages of glucose.

The ability to hydrolyze and saccharify starch that is raw or only partially pasted represents an important energy savings. Pasting is generally carried out at temperatures of 100° C. to 130° C. and upwards, therefore requiring significant input of thermal energy. RSH enzyme preparations, on the other hand, catalyze starch saccharification efficiently at relatively low temperatures (e.g., at 65° C. or less) and there is no need to heat the starch at any time to higher temperatures. However, starch slurries that are to be hydrolyzed with RSH enzyme preparations may be initially swelled at somewhat higher temperatures (e.g., 65° C. to 100° C.) to hasten the hydrolyzing process. Such a swelling procedure may be considered a partial solubilization and pasting step; nevertheless, starch subjected to these temperatures for short periods of time remains substantially in granular form.

It would be desirable for several reasons to use RSH enzyme preparations to solubilize and saccharify starch slurried at higher solids levels (e.g., between 20 and 60 weight percent starch, and more particularly between about 25 and about 40 weight percent starch, d.s.) than the 15% d.s. level that may generally be hydrolyzed by certain RSH enzyme preparations in a one-step reaction. Higher starch content slurries require less processing reactor tank space. The resulting saccharide solution or syrup is more highly concentrated, and thus, less energy is required for removing water therefrom to further concentrate or dry the same. When slurries higher than 15% solids are hydrolyzed with RSH enzyme preparations, a more concentrated saccharide solution is typically produced; however, in a reaction

conducted for a reasonable period of time, a substantial amount of the starch remains insoluble, and unless this remaining starch can also be solubilized or otherwise used, the unsolubilized starch represents a substantial economic loss.

When high starch concentration slurries are enzymatically hydrolyzed with RSH enzyme preparations, hydrolysis generally proceeds at an appreciable rate only for a certain time period, typically about 48 hours. After this, the rate of hydrolysis drops off considerably. With glucose being produced at lower rates, it is an inefficient utilization of reactor tank space to allow the reaction to continue. Perhaps more importantly, however, with high glucose concentrations, a significant amount of undesired disaccharide formation occurs and such disaccharides are difficult to hydrolyze. Thus the percent of glucose in the syrup decreases and the percent of higher saccharides increases. Such products are undesirable. For example, if glucose syrup is converted to a high fructose syrup, the higher saccharides do not contribute to the sweetness and may even detract from the sweetness. For these reasons, high solids starch slurries cannot generally be satisfactorily hydrolyzed by RSH enzyme preparations in a single step.

Raw starch, such as raw corn starch, contains in addition to the starch, fiber, protein and fat, which comprise an insoluble sludge or "mud" upon partial solubilization and saccharification of the starch. As the mud builds up in the process system, efficiency of enzymatic starch hydrolysis diminishes. As such, it is not feasible to produce highly concentrated syrups by a simple process of continuously adding additional starch slurry to a reaction vessel as syrup is continuously or periodically removed therefrom. Accordingly, the present invention seeks to solubilize and saccharify high solids granular starch slurries and to recover in solubilized and saccharified form as much starch as possible from the insoluble mud.

It is a primary object of the present invention to provide an economical process which converts a slurry of high solids granular starch substantially completely to glucose (dextrose) at a low temperature. It is a further object of the present invention to provide such a process which facilitates removal of insoluble "mud" (fiber, protein and fat) from the predominantly glucose syrup which is produced.

#### SUMMARY OF THE INVENTION

An aqueous slurry of milled granular starch of between about 20 and 60 weight percent starch, dry substance (d.s.), is converted in a multi-step process substantially entirely to a syrup containing a high percentage of glucose using raw starch hydrolyzing (RSH) enzyme preparations. In a first step, the enzyme preparation is added to a first starch slurry and allowed to act for a period of time sufficient to solubilize and saccharify at least a substantial portion (e.g., at least about 25, preferably at least about 50 and most preferably at least about 60 percent), but less than all, of the starch contained in said first starch slurry. The reaction is preferably terminated before the percent glucose in the solubilized saccharide product, d.s., drops to about 95 percent, and this will usually occur when the conversion of starch is below about 95 percent. The solubilized saccharide product or syrup of the first step, which generally will contain at least 22% d.s. saccharides, is separated from insolubles which yet contains substantial amounts of unsolubilized starch. The insolubles are

reslurried to form a second slurry having a starch concentration of less than 20 percent by weight d.s. The starch in said second slurry is then hydrolyzed by contacting same with an RSH enzyme preparation for a time sufficient to solubilize and saccharify substantially all of the starch contained in said second slurry. The remaining insolubles, containing fiber, protein and fat, are removed by centrifugation, filtration or other suitable separation means, from the syrup prepared in the second step. The syrup from the second step of the process can be pooled, if desired, with that from the first step and, in that event, the resulting pooled syrup will generally contain upwards of 20 percent by weight saccharides, at least 95% of which is glucose.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In accordance with the present invention, slurries of 20 to 60 (preferably from about 25 to about 40) weight percent granular starch are substantially completely solubilized and saccharified with an RSH enzyme preparation in a multi-step process to produce a pooled syrup with upwards of 20% by weight saccharides, with at least 95 percent by weight of said saccharides being glucose. In the first step of the process, the pH and temperature of the slurry are adjusted to values suitable to promote RSH activity, and an RSH enzyme preparation of sufficient activity to promote a relatively rapid rate of starch solubilization and saccharification is added. Preferably, the reaction is allowed to proceed until at least a substantial portion, but less than all, of the starch is solubilized and the weight percent of solubilized saccharides, d.s., is at least about 22%. The resulting solution or syrup is separated, e.g., by centrifugation, filtration, etc., from the insolubles which contain the fiber, protein and fat along with a significant amount of starch that has not been solubilized in the initial step. This separation is preferably made before the glucose percentage of the solubilized saccharide drops below 95% (and most preferably before said glucose percentage drops below 97%) as it would if the first stage hydrolysis is allowed to continue overly long. The first step is generally complete within 48 hours.

In the second step, the insolubles are mixed with additional water to form a second slurry which contains less than 20% by weight, d.s., insoluble starch and preferably about 16% starch or less. Conditions of temperature and pH are adjusted to promote RSH preparation activity. Conversion of the starch is allowed to proceed in the presence of an RSH enzyme preparation until very little (e.g., generally less than about 10%, preferably less than about 5% and most preferably less than about 1%) of the starch used to form the second slurry remains unsolubilized. Any starch remaining unsolubilized following the second step hydrolysis remains with mud which is released from the starch during hydrolysis and which is comprised of fiber, protein and fat. Preferably, the syrup produced in this second step is at least 15 percent by weight saccharide, d.s. Typically, the syrups from the two steps are pooled, and if so pooled, provide a syrup which is at least about 20 weight percent saccharides, d.s. The percent of the saccharides which is glucose as a result of each of the first and second steps, individually, is preferably at least about 95 percent by weight.

The amount of starch which can be solubilized within a commercially acceptable time period in the first step before the reaction is terminated varies according to the

percentage of starch in the initial slurry. For example, if the initial slurry is about 36 percent starch, only about 60 percent of the starch will generally be solubilized in the first step within a 48-hour hydrolysis period, whereas if the initial slurry is about 26 percent starch, upwards of about 85 percent of the starch is solubilized in the first step within the same hydrolysis period. The higher the starch content of the initial starch slurry, the higher the concentration of saccharide in the syrup resulting from the first step. For example, with a 36 percent starch slurry, saccharide solutions of up to about 29 percent d.s. saccharide content may be obtained in the first step without the percent glucose in the syrup dropping below 95%, whereas with a 26 percent starch slurry, saccharide solutions of about 24 percent d.s. saccharide content may be obtained without the percent glucose in the syrup dropping below 95%. In the second step, the slurry is preferably adjusted to about the highest starch percent consistent with substantially complete solubilization of the remaining starch, e.g., up to about 20 percent and preferably about 16 percent. The degree of starch solubilization and saccharide content of the syrup produced in the second step at a given second step slurry starch solids content will be substantially the same, regardless of the percent of starch used to form the initial slurry in the first step of the process. In the second step within a period of about 48 hours, in excess of 95% of the remaining starch will typically be hydrolyzed and generally in excess of 99% thereof will be hydrolyzed.

An important advantage of the invention is that the relatively high starch solids granular starch slurries which are solubilized in the first step are contained in much smaller reactor vessels than would be required if initial slurries were of a starch percentage capable of being solubilized in a single step, i.e. at less than 20 weight percent starch solids. Furthermore, much more highly concentrated syrups are produced than can be obtained from a one-step solubilization of low solids starch slurries, thereby avoiding the need for concentrating as extensively. The second step corresponds, both in initial starch concentration and in saccharide solution produced, to that of a less than 20 weight percent starch solids, one-step solubilization, but of course, at this step of the overall process, the major quantity of starch which was present in the initial starch slurry has already been solubilized and removed as syrup. In total, the entire two-step process requires substantially less reactor vessel volume and produces a pooled syrup of significantly higher concentration than can be obtained in a one-step conversion of low solids starch slurry. In a multi-step process using initial starch slurries of 20 to 60 percent, relatively little starch is lost to the fiber, protein and fat residue (i.e., "mud"), whereas in a single step process beginning with the same high starch solids level, significant quantities of starch would be lost to the mud.

Enzyme systems or preparations which are generally suitable for use in the practice of the present invention include any of those exhibiting raw starch hydrolyzing (RSH) activity or capability (i.e., RSH enzyme preparations). However, the advantages of the present invention (e.g., relative to a single step, high starch solids aqueous slurry enzyme hydrolysis process) are most pronounced in the case of those RSH enzyme preparations whose solubilization rates tend to decrease substantially in the later stages (e.g., after 50 to 60% starch solubilization) of relatively high starch solids (e.g., in

excess of 20 weight percent starch contents on a total slurry weight basis), single step hydrolysis operations and/or which tend to exhibit undesirably high levels (e.g., 5 weight percent or more on a solubilized saccharide total weight basis) of di-, tri- and higher polysaccharide formation when said relatively high starch solid, single stage hydrolysis operations are carried to relatively high degrees (e.g., 95 or more) of starch conversion. Accordingly, the application or use of the present invention in conjunction with this latter type of RSH enzyme is particularly beneficial and preferred.

A particularly preferred RSH enzyme preparation for use in the present invention is the hereinbefore described *Humicola* RSH enzyme preparation. Said *Humicola* RSH enzyme preparation exhibits maximum activity within the pH range of 5.0 to 7.0 and particularly in the range of 5.5 to 6.0. Said enzyme preparation exhibits maximum activity in the temperature range of 50° C. to 65° C. In each of the steps, the enzymatic solubilization of starch is preferably carried out within these pH and temperature ranges.

Certain especially preferred *Humicola* RSH enzyme preparations for use in the present invention are those obtained from a fungal organism strain of *Humicola grisea* var. *thermolidea* selected from the group consisting of ATCC 16453; NRRL 15219; NRRL 15220; NRRL 15221; NRRL 15222; NRRL 15223; NRRL 15224 and NRRL 15225 (and genetically altered strains artificially derived therefrom), wherein the ATCC number represents a Deposit Type Collection Number of the American Type Culture Collection Depository in Rockville, Md. and wherein "NRRL" stands for the USDA's Northern Regional Research Laboratory in Peoria, Ill.

As noted above, *Humicola* RSH enzyme preparation contains glucoamylase activity as well as a potentiating factor which solubilizes raw starch. The relative proportions of potentiating factor and glucoamylase activity in other RSH enzyme preparations may vary somewhat. However, with RSH enzyme preparations of interest for use in the practice of the present invention, there is usually ample potentiating factor produced along with the glucoamylase fraction. Accordingly, for purposes of this invention, the activity of the RSH enzyme preparations is defined in terms of their glucoamylase activity.

Glucoamylase activity is measured for purposes of this invention in 10 D.E. units for either RSH enzyme preparation or conventional glucoamylase. A 10 D.E. unit is the amount of either type of enzyme which produces 1 micromole of glucose per minute under the assay conditions. To determine glucoamylase activity for purposes of this invention, one-tenth ml of enzyme preparation, diluted if necessary, containing 0.06 units to 1.1 units is added to 0.9 ml of substrate solution preheated at 50° C. for 5 minutes. The substrate solution consists of 40 parts by volume 0.25M sodium acetate buffer (pH 5.5) and 50 parts by volume 4% by weight 10 D.E. maltodextrin in water. The substrate solution is kept at 50° C. for 5 minutes before the enzyme solution is added. After 10 minutes, the reaction is quenched by pouring into a preheated 16 mm test tube and heating in a 100° C. water bath for 6 minutes. Glucose concentration is determined by any convenient method, such as glucose reagent kit No. 15-UV from Sigma Chemical Co. or with an instrument such as the Technicon Auto-analyzer.

In the first starch solubilization and saccharification step of the present invention, the RSH enzyme preparation is employed in an amount sufficient to solubilize at least a substantial portion (and preferably a major proportion) of the granular starch contained within said first starch slurry. Typically, the addition of RSH enzyme preparation to the first starch slurry in an amount corresponding to from about 2 to about 60 (preferably from about 5 to about 30) 10 D.E. units per gram of starch in said first slurry is adequate for such purpose.

In the second starch solubilization and saccharification step of the invention, the RSH enzyme preparation is employed in an amount sufficient to solubilize substantially all (e.g., in excess of 90%, preferably at least about 95%, more preferably at least about 97% and most preferably about 99% or more) of the starch contained within the second starch slurry. Here again, from about 2 to about 60 (preferably about 5 to about 30) 10 D.E. units per gram of starch in said second slurry will suffice for such purpose.

With regard to the foregoing, it should be noted that RSH enzyme preparation from the first saccharification step will oftentimes be carried over into the second starch slurry in the mud/unsolubilized starch residue from said first stage solubilization. Accordingly, the amount of RSH enzyme preparation which needs to be freshly or separately added to said second slurry to obtain the requisite second slurry enzyme content or activity may be substantially less than the numerical 10 D.E. unit ranges stated above. Indeed, in some instances, the amount of RSH enzyme preparation carried over from the first solubilization may, by itself, be totally sufficient for the second slurry solubilization step. In such instances, no separate or fresh addition of RSH enzyme preparation is required in connection with the second stage solubilization reaction.

The RSH enzyme preparation may be the unpurified broth produced, for example, by fermentation of a fungus. Alternatively, the RSH enzyme preparation may be purified to various extents to remove inactive protein. The active components of RSH enzyme preparation are not adsorbed by DEAE cellulose, e.g., Whatman pre-swollen Microgranular Anion Exchanger DE52 diethylaminoethyl cellulose, and inactive proteins are removed by adsorption to the cellulose. When exposed either to a column or bath of DEAE cellulose at a pH of 5.0 to 7.0 (preferably 6.5 to 7.0), a 2 to 4.5 fold increase in specific activity is achieved which is attributable at least in part to the removal of inactive proteins.

The relative rates of solubilization and glucose production are affected by the concentrations of calcium ion in the reaction mixture. For example, calcium at levels of about 10 to 200 ppm and preferably at levels of 30 to 100 ppm promotes RSH enzyme preparation activity and stabilizes the same with respect to slightly higher temperatures. Higher calcium levels may decrease RSH enzyme preparation activity.

Practically any starch source is suitable for the method of the present invention as RSH enzyme preparations will solubilize and saccharify all starches, both of the amylose and amylopectin varieties. However, due to practical considerations such as cost, availability, etc., corn starch represents a particularly preferred starch for use in the practice of the present invention. There is no need to specially prepare the starch, and ground whole kernel corn is a suitable corn starch source, as is degerminated yellow corn meal.

The invention will now be described in greater detail by way of the following specific examples in which all parts and percentages are on a weight basis and temperatures are in degrees Celsius unless otherwise indicated.

#### EXAMPLE 1

Corn starch was slurried in water at 26% d.s. starch. Calcium was added to a concentration of 30 ppm; the pH was adjusted to 5.7; and the temperature was raised to 55° C. 15 10 D.E. units of unrefined RSH enzyme preparation obtained from the fermentation broth of a mutant strain of the fungus *Humicola grisea* var. *thermoidea* (ATCC 16453) per gram of starch was added. The reaction proceeded at 55° C. for 48 hours as the slurry was stirred. About 82 percent of the initially charged starch was solubilized in this first hydrolysis step.

The slurry was filtered to separate the syrup from the insolubles and the phases were separated. The syrup was 24.0% saccharides of which 97.4% was glucose on a dry substance basis.

The insolubles were reslurried at 16% starch, d.s. and the conditions adjusted as above, including the addition of an additional 15 10 D.E. units per gm. of starch. The reaction proceeded for an additional 48 hours, after which the remaining insolubles were separated from the syrup by centrifugation. The syrup from the second reaction was 17.5% saccharides, of which 96.4%, on a dry substance basis, was glucose. The syrups were pooled producing a blended syrup product which was 22.9% saccharides, of which 97.2%, on a dry substance basis, was glucose.

The insoluble mud from centrifugation contained less than 1% of the starch initially present.

#### EXAMPLE 2

Corn starch was slurried in water at 36% d.s. starch. Calcium was added to a concentration of 30 ppm; the pH was adjusted to 5.7; and the temperature was raised to 55° C. 15 10 D.E. units of unrefined RSH preparation obtained from the fermentation broth of a mutant strain of the fungus *Humicola grisea* var. *thermoidea* (ATCC 16453) per gram of starch was added. The reaction proceeded at 55° C. for 48 hours as the slurry was stirred. About 64 percent of the initially charged starch material was solubilized in this first hydrolysis step.

The slurry was filtered to separate the syrup from the insolubles and the phases were separated. The syrup was 28.5% saccharides, 96.5% of which was glucose on a dry substance basis.

The insolubles were reslurried at 16% starch, d.s., and the conditions adjusted as above, including the addition of an additional 15 10 D.E. units per gm. of starch. The reaction proceeded for an additional 48 hours, after which the remaining insolubles were separated from the syrup by centrifugation. The syrup from the second reaction was 17.5% saccharides, of which 97.5% was glucose on a dry substance basis. The syrups were pooled, producing a blended syrup product which was 24.6% saccharides, of which 96.9%, on a dry substance basis, was glucose.

The insoluble mud contained less than 1% of the starch initially present.

The above examples demonstrate that excellent results are achieved through the use of enzyme with RSH activity in accordance with the present invention. In the process, substantially all of the starch is solubilized and saccharified. The combined syrups are higher than

about 22% (preferably higher than about 24%) saccharide, at least about 95% (preferably at least about 96%) of which is glucose. The syrups are ideal stock for enzymatic conversion to high fructose syrup or fermentation to ethanol. Being high in saccharide content, the syrups may be concentrated or dried with less energy. When even higher initial saccharide concentrations are desired, the syrup from the first step may be used alone and the syrup from the second step used for other purposes.

The enzymatic conversion is carried out on raw starch at a relatively low temperature and avoids energy-intensive pasting. The process requires few pH adjustments and minimal additional costs.

While the invention has been described in terms of certain preferred embodiments, modifications obvious to one with ordinary skill in the art may be made without departing from the scope of the invention. For instance, while conducting the present invention in only two distinct starch slurry solubilization stages is satisfactory for most purposes and is, indeed, preferred for the sake of simplicity, those skilled in the art will, of course, recognize that the use of three or more separate and distinct starch slurry solubilization stages can also be satisfactorily employed to reap the benefits of the present invention and without departing from the spirit and scope thereof.

The present invention and various embodiments thereof are set forth in the following claims.

What is claimed is:

1. A method of preparing a syrup containing, on a dry substance weight basis, a high percentage of glucose, said method comprising:
  - (a) preparing a first aqueous slurry of granular starch containing between about 20 and about 60 weight percent starch, d.s.;
  - (b) adjusting the pH and the temperature of said first slurry to values suitable to promote activity of a raw starch hydrolyzing enzyme preparation which is produced by a fungal organism and which catalyzes the hydrolysis of granular starch directly to glucose, said raw starch hydrolyzing enzyme preparation being characterized in that it catalyzes the hydrolysis of granular starch suspended in water at a concentration of about 15 percent by weight starch solids substantially completely to soluble glucose syrup solids containing at least about 97 percent by weight glucose, d.s., when the hydrolysis is carried out at a pH of about 5.0 to about 7.0 and a temperature of about 55° C. and without added alpha amylase or added debranching enzyme of the pullulanase, isoamylase or beta amylase type, said enzyme preparation being further characterized in that it is separable by carboxymethyl cellulose into a first adsorbing fraction and a second non-adsorbing proteinaceous fraction, said first adsorbing fraction containing a glucoamylase enzyme (EC 3.2.1.3) that has an isoelectric point of about 8.0 or higher and said second non-adsorbing fraction having glucoamylase-potentiating activity that catalyzes the hydrolysis of granular starch;
  - (c) adding to said first slurry the raw starch hydrolyzing enzyme preparation in an amount sufficient to solubilize and saccharify a substantial portion of the starch in said first slurry;
  - (d) allowing said raw starch hydrolyzing enzyme preparation to solubilize and saccharify a substan-

tial portion, but less than all, of the starch in said first slurry to yield a first syrup of at least about 22 weight percent saccharides, d.s.;

- (e) separating said first syrup from the insolubles which remain and which contain unsolubilized starch from said first slurry;
  - (f) preparing a second aqueous slurry from said insolubles, said second slurry containing less than about 20 weight percent, d.s., starch;
  - (g) adjusting the pH and the temperature of said second slurry to values suitable to promote raw starch hydrolyzing enzyme activity;
  - (h) contacting said second slurry with the raw starch hydrolyzing enzyme preparation in an amount sufficient to solubilize and saccharify substantially all of the starch contained in said second slurry;
  - (i) allowing said raw starch hydrolyzing enzyme preparation to solubilize and saccharify substantially all of the starch in said second slurry to provide a second syrup; and
  - (j) separating said second syrup from insolubles contained therein.
2. A method according to claim 1 wherein the step (e) separation is made before the glucose content of the first syrup from step (d) drops below the 95 weight percent of the soluble saccharides in said first syrup.
  3. A method according to claim 1 wherein step (d) is conducted over a period of about 48 hours or less.
  4. A method according to claim 1 wherein said insolubles are removed from said syrups in steps (e) and (j) by centrifugation.
  5. A method according to claim 1 wherein step (i) is carried out until at least about 95% of the starch in said second slurry is solubilized.
  6. A method according to claim 1 wherein each of said first and second slurries contains between about 10 and about 200 ppm calcium.
  7. A method according to claim 1 wherein said step (d) is conducted until at least about 25 percent of the starch in said first slurry is solubilized and saccharified.
  8. The method according to claim 1 wherein said step (d) is conducted until at least about 50 percent of the starch in said first slurry is solubilized and saccharified.
  9. The method according to claim 1 wherein at least about 60 percent of the starch in said first slurry is solubilized and saccharified in step (d) thereof.
  10. The method according to claim 1 wherein said insolubles are removed from the syrups in steps (e) and (j) by centrifugation or filtration.
  11. The method according to claim 1 wherein the first aqueous granular starch slurry contains from about 25 to about 40 weight percent granular starch on a total slurry weight basis.
  12. The method according to claim 1 wherein the starch slurry of step (f) contains about 16 weight percent or less of granular starch on a total slurry weight basis.
  13. The method of claim 1 wherein step (d) is terminated before the glucose content of the first syrup drops below about 97 weight percent on a soluble saccharide weight basis.
  14. The method of claim 1 wherein said first and second syrups are pooled to provide a combined syrup containing in excess of 22 weight percent solubilized saccharide on a total syrup weight basis and in which at least about 95 weight percent of said saccharide is glucose.

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15. The method of claim 1 wherein said first and second syrups are pooled to provide a combined syrup containing in excess of 24 weight percent solubilized saccharide on a total syrup weight basis, of which at least about 96 weight percent is glucose.

16. The method of claim 1 wherein the solubilization and saccharification of said first slurry is conducted at a pH of from about 5.0 to about 7.0 and at a temperature of from about 50° to about 65° C.

17. The method of claim 16 wherein the solubilization and saccharification of said second slurry is conducted at a pH of from about 5.0 to about 7.0 and at a temperature of from about 50° to about 65° C.

18. The method of claim 17 wherein the enzyme employed for the solubilization and saccharification of both the first and second starch slurries is a *Humicola* RSH enzyme.

19. The method of claim 1 wherein from about 2 to about 60 10 D.E. units of said RSH enzyme per gram of starch is employed to solubilize and saccharify each of said first and second aqueous starch slurries.

20. The method of claim 1 wherein the amount of RSH enzyme employed to solubilize and saccharify each of said first and second aqueous starch slurries is between about 5 and about 30 10 D.E. units.

21. The method of claim 1 wherein the granular starch employed is derived from corn.

22. A method of preparing a syrup containing, on a dry substance weight basis, a high percentage of glucose, said method comprising:

- (a) preparing a first aqueous slurry of granular starch containing between about 20 and about 60 weight percent starch, d.s.;
- (b) adjusting the pH and the temperature of said first slurry to values suitable to promote activity of a raw starch hydrolyzing enzyme preparation which is produced by a fungal organism that is a strain of the genus *Humicola* and which catalyzes the hydrolysis of granular starch directly to glucose, said raw starch hydrolyzing enzyme preparation being characterized in that it catalyzes the hydrolysis of granular starch suspended in water at a concentration of about 15 percent by weight starch solids substantially completely to soluble glucose syrup solids containing at least about 97 percent by weight glucose, d.s., when the hydrolysis is carried out at a pH of about 5.0 to about 7.0 and a temperature of about 55° C. and without added alpha amylase or added debranching enzyme of the pullulanase, isoamylase or beta amylase type, said enzyme preparation being further characterized in that it is separable by carboxymethyl cellulose into a first adsorbing fraction and a second non-adsorbing proteinaceous fraction, said first adsorbing fraction containing a glucoamylase enzyme (EC 3.2.1.3) that has an isoelectric point of about 8.0 or higher and said second non-adsorbing fraction having glucoamylase-protentiating activity that catalyzes the hydrolysis of granular starch;
- (c) adding to said first slurry the raw starch hydrolyzing enzyme preparation in an amount sufficient to solubilize and saccharify a substantial portion of the starch in said first slurry;
- (d) allowing said raw starch hydrolyzing enzyme preparation to solubilize and saccharify a substantial portion, but less than all, of the starch in said first slurry to yield a first syrup of at least about 22 weight percent saccharides, d.s.;

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(e) separating said first syrup from the insolubles which remain and which contain unsolubilized starch from said first slurry;

(f) preparing a second aqueous slurry from said insolubles, said second slurry containing less than about 20 weight percent, d.s., starch;

(g) adjusting the pH and the temperature of said second slurry to values suitable to promote raw starch hydrolyzing enzyme activity;

(h) contacting said second slurry with the raw starch hydrolyzing enzyme preparation in an amount sufficient to solubilize and saccharify substantially all of the starch contained in said second slurry;

(i) allowing said raw starch hydrolyzing enzyme preparation to solubilize and saccharify substantially all of the starch in said second slurry to provide a second syrup; and

(j) separating said second syrup from insolubles contained therein.

23. A method of preparing a syrup containing, on a dry substance weight basis, a high percentage of glucose, said method comprising:

(a) preparing a first aqueous slurry of granular starch containing between about 20 and about 60 weight percent starch, d.s.;

(b) adjusting the pH and the temperature of said first slurry to values suitable to promote activity of a raw starch hydrolyzing enzyme preparation which is produced by a fungal organism that is a strain of the species *Humicola grisea* and which catalyzes the hydrolysis of granular starch directly to glucose, said raw starch hydrolyzing enzyme preparation being characterized in that it catalyzes the hydrolysis of granular starch suspended in water at a concentration of about 15 percent by weight starch solids substantially completely to soluble glucose syrup solids containing at least about 97 percent by weight glucose, d.s., when the hydrolysis is carried out at a pH of about 5.0 to about 7.0 and a temperature of about 55° C. and without added alpha amylase or added debranching enzyme of the pullulanase, isoamylase or beta amylase type, said enzyme preparation being further characterized in that it is separable by carboxymethyl cellulose into a first adsorbing fraction and a second non-adsorbing proteinaceous fraction, said first adsorbing fraction containing a glucoamylase enzyme (EC 3.2.1.3) that has an isoelectric point of about 8.0 or higher and said second non-adsorbing fraction having glucoamylase-protentiating activity that catalyzes the hydrolysis of granular starch;

(c) adding to said first slurry the raw starch hydrolyzing enzyme preparation in an amount sufficient to solubilize and saccharify a substantial portion of the starch in said first slurry;

(d) allowing said raw starch hydrolyzing enzyme preparation to solubilize and saccharify a substantial portion, but less than all, of the starch in said first slurry to yield a first syrup of at least about 22 weight percent saccharides, d.s.;

(e) separating said first syrup from the insolubles which remain and which contain unsolubilized starch from said first slurry;

(f) preparing a second aqueous slurry from said insolubles, said second slurry containing less than about 20 weight percent, d.s., starch;

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- (g) adjusting the pH and the temperature of said second slurry to values suitable to promote raw starch hydrolyzing enzyme activity;
  - (h) contacting said second slurry with the raw starch hydrolyzing enzyme preparation in an amount sufficient to solubilize and saccharify substantially all of the starch contained in said second slurry;
  - (i) allowing said raw starch hydrolyzing enzyme preparation to solubilize and saccharify substantially all of the starch in said second slurry to provide a second syrup; and
  - (j) separating said syrup from insolubles contained therein.
24. A method of preparing a syrup containing, on a dry substance weight basis, a high percentage of glucose, said method comprising:
- (a) preparing a first aqueous slurry of granular starch containing between about 20 and about 60 weight percent starch, d.s.;
  - (b) adjusting the pH and the temperature of said first slurry to values suitable to promote activity of a raw starch hydrolyzing enzyme preparation which is produced by a fungal organism that is a strain of the species *Humicola grisea* var. *thermoidea* and which catalyzes the hydrolysis of granular starch directly to glucose, said raw starch hydrolyzing enzyme preparation being characterized in that it catalyzes the hydrolysis of granular starch suspended in water at a concentration of about 15 percent by weight starch solids substantially completely to soluble glucose syrup solids containing at least about 97 percent by weight glucose, d.s., when the hydrolysis is carried out at a pH of about 5.0 to about 7.0 and a temperature to about 55° C. and without added alpha amylase or added debranching enzyme of the pullulanase, isoamylase or beta amylase type, said enzyme preparation being further characterized in that it is separable by car-

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- boxymethyl cellulose into a first adsorbing fraction and a second non-adsorbing proteinaceous fraction, said first adsorbing fraction containing a glucoamylase enzyme (EC 3.2.1.3) that has an isoelectric point of about 8.0 or higher and said second non-adsorbing fraction having glucoamylase-protentiating activity that catalyzes the hydrolysis of granular starch;
  - (c) adding to said first slurry the raw starch hydrolyzing enzyme preparation in an amount sufficient to solubilize and saccharify a substantial portion of the starch in said first slurry;
  - (d) allowing said raw starch hydrolyzing enzyme preparation to solubilize and saccharify a substantial portion, but less than all, of the starch in said first slurry to yield a first syrup of at least about 22 weight percent saccharides, d.s.;
  - (e) separating said first syrup from the insolubles which remain and which contain unsolubilized starch from said first slurry;
  - (f) preparing a second aqueous slurry from said insolubles, said second slurry containing less than about 20 weight percent, d.s., starch;
  - (g) adjusting the pH and the temperature of said second slurry to values suitable to promote raw starch hydrolyzing enzyme activity;
  - (h) contacting said second slurry with the raw starch hydrolyzing enzyme preparation in an amount sufficient to solubilize and saccharify substantially all of the starch contained in said second slurry;
  - (i) allowing said raw starch hydrolyzing enzyme preparation to solubilize and saccharify substantially all of the starch in said second slurry to provide a second syrup; and
  - (j) separating said second syrup from insolubles contained therein.
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[54] METHOD FOR DIRECT  
SACCHARIFICATION OF RAW STARCH  
USING ENZYME PRODUCED BY A  
BASIDIOMYCETE BELONGING TO THE  
GENUS CORTICIUM

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C12N 9/30; C12N 9/34; C12R 1/645

[52] U.S. Cl. .... 435/96; 435/99;  
435/203; 435/205; 435/911

[58] Field of Search ..... 435/96, 99, 203, 205

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McClelland & Maier

[57] ABSTRACT

A method for the cooking-free saccharification of starch using an amylase produced by *Corticium rolfsii* AHU 9627 or its variants. According to the method, even a high viscous suspension of 10% (w/v) or more raw-corn starch is almost completely hydrolyzed within 8 hours. The saccharification is proceeded at a higher temperature and a lower pH compared with those in known methods utilizing other amylases which are able to hydrolyze uncooked starch, so that the propagation of the infectious bacteria which would affect the saccharifying efficiency can be avoided.

2 Claims, 2 Drawing Figures

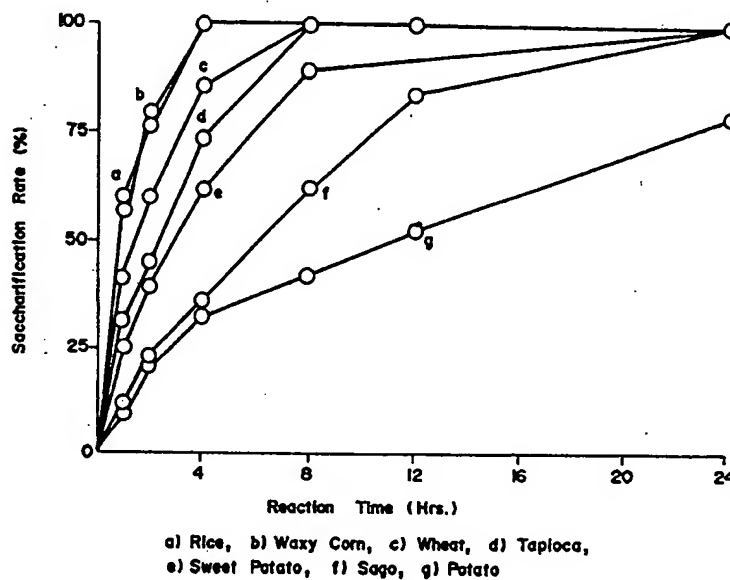


FIG. 1

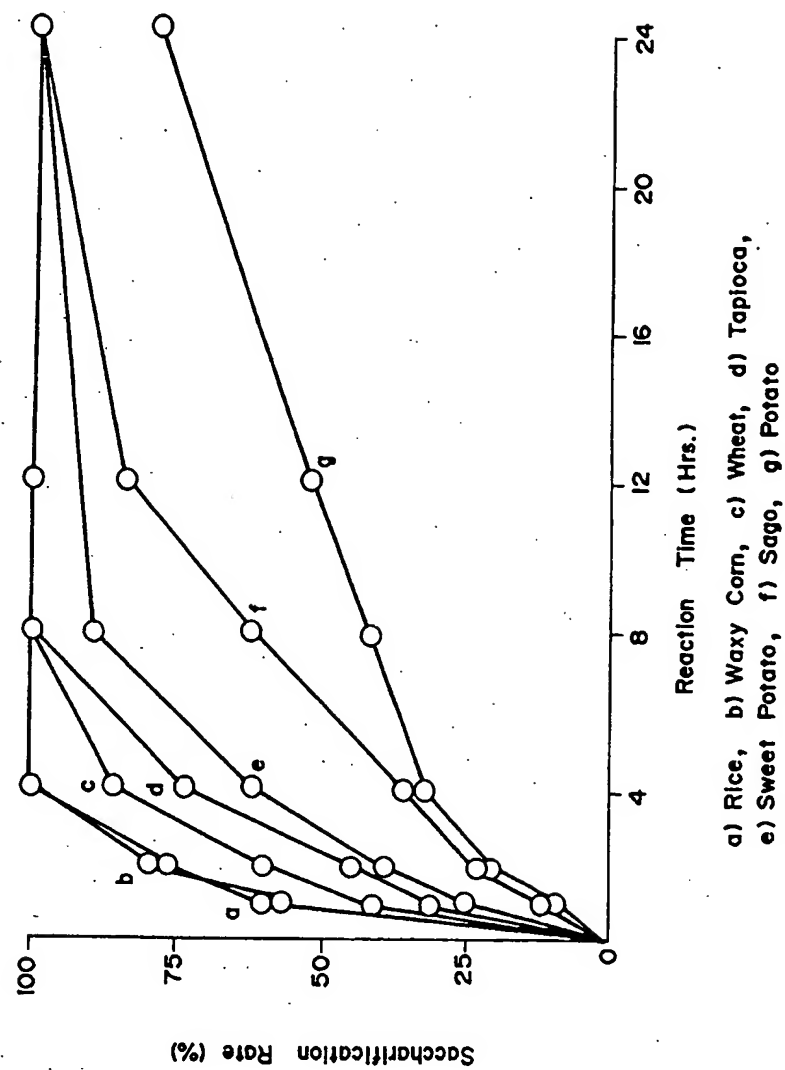
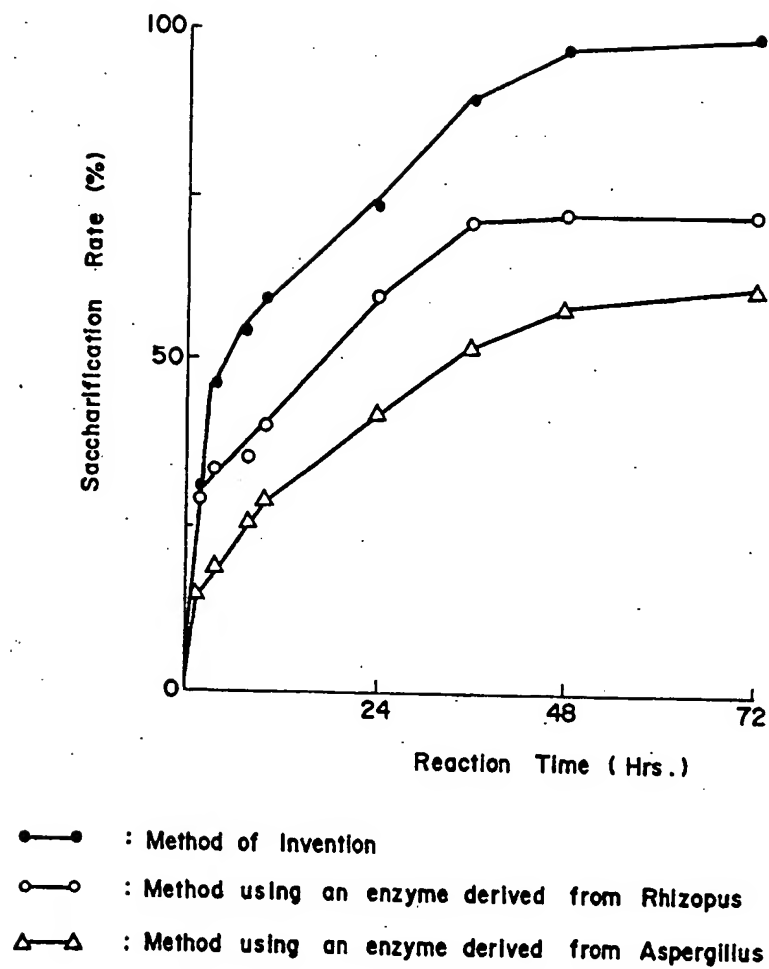


FIG. 2



# METHOD FOR DIRECT SACCHARIFICATION OF RAW STARCH USING ENZYME PRODUCED BY A BASIDIOMYCETE BELONGING TO THE GENUS CORTICIUM

## BACKGROUND OF THE INVENTION

### (1) Field of the Invention

This invention relates to the saccharification of starchy substances, particularly to the method for glucose production from raw-starch using the enzyme produced by a basidiomycete belonging to the genus *Corticium*.

### (2) Description of the Prior Art

On the manufacturing process of glucose, starchy substances have been saccharified by two-step reactions using two different types of amylases. The process consists of cooking process of starchy substances at elevated temperature, liquefaction of cooked starch by  $\alpha$ -amylase at a temperature between 80° and 120° C., and saccharification by glucoamylase at a temperature between 45° and 60° C.

This method requires a large amount of thermal energy in the preceding cooking process and some complicated procedures because the optimum temperatures of  $\alpha$ -amylase and glucoamylase, and the optimum pHs of these enzymes are different, respectively. Furthermore, the cooked starch is so pasty that the enzyme reaction do not proceed uniformly at the concentration between 30 and 50%. From the standpoint of the control of factory, the process involves many difficult problems to be solved.

In order to overcome the above mentioned drawback, it has been made many attempts to saccharify raw-starch without cooking. For this purpose, the enzymes produced by the fungi belonging to the genus *Aspergillus* and the genus *Rhizopus* were frequently used. In these methods, glucose can be obtained by one-step reaction and the reaction mixture shows low viscosity even at a higher concentration of starch than 30%. Therefore, the methods provide some advantages in energy cost and operation techniques.

However, most enzymes used in these processes have less activity toward raw starch at a high substrate concentration. According to the report by Ueda et al on the enzymes of *Aspergillus awamori* [Starch, 33(9), 313(1981)] and *Rhizopus* sp. [Starch, 27, 123(1975)], an upper limit of starch concentration for the saccharification is 2% at the most. Moreover, the enzyme produced by *Chalara paradoxa*, which was reported by Kainuma et al as an active enzyme capable of saccharifying raw starch ["Dempun Kagaku" (Starch Science), 32(3), 189(1985) and Japanese Laid-open patent application No. 59-140896] was able to hydrolyze raw starch only at a concentration less than 5%. These results suggest that enzymatic saccharification of raw starch at a concentration more than 10% is very difficult.

In order to saccharify uncooked starchy substances, attention should be paid to prevent the contamination of various microbials during the reaction. The reaction should be carried out at a temperature as high as possible, preferably at a temperature between 45° and 60° C. The enzymes produced by *Rhizopus* sp. and *Chalara paradoxa* are inadequate for the saccharification of raw-starch, because they cannot be used at a temperature higher than 50° C.

In recent years, it has been reported that an enzyme produced by a strain of *Corticium rolsii* (IFO 4878) has

some saccharifying activity toward uncooked starch [Nippon Shokuhin Kogyo Gakkaishi, 25 (1), 22 (1978)]. This enzyme has the activity to saccharify about 90% of starch in the reaction mixture containing 10% corn starch (w/v). As they stated in the report, it is believed that there is a limit to achieve effective saccharification of uncooked starch by the enzyme.

As it has been verified by the above mentioned references, there has never been a suitable method for industrial saccharification of starch without cooking.

## SUMMARY OF THE INVENTION

The present inventors made extensive studies on the method for the enzymatic saccharification of starch without cooking. As a result, it was found that the enzyme produced by a fungus belonging to the genus *Corticium* had much higher activity toward uncooked starch than known glucoamylases, and a suspension of 10% (w/v) raw-corn starch was almost completely hydrolyzed by the enzyme within 8 hours. It was also found that the saccharification proceeded at a higher temperature and a lower pH than the other amylases which were able to hydrolyze uncooked starch. These properties are very profitable from the standpoint of controlling the infectious bacteria which would effect the saccharifying efficiency.

Particularly, the present invention provides a method for the cooking-free saccharification of starch by an amylase produced by *Corticium rolsii* AHU 9627 or its variants.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graph showing the relation between saccharification rate and reaction time, when various kinds of raw-starch were saccharified by the invented method (Example 1).

FIG. 2 is a graph showing the relation between saccharification rate and reaction time, when raw-corn starch was saccharified by various kinds of enzymes (Example 2).

## DETAILED DESCRIPTION OF THE INVENTION AND PREFERRED EMBODIMENTS

The strain (AHU 9627) used in the present invention, which is able to produce an active amylase capable of saccharifying raw-starch, has the following mycological properties.

### (1) Growth on Various Media

(i) Growth on malt extract agar and oat meal agar is very good. Colonies reach about 9 cm in diameter in 7 days at 30° C. Plumose white mycelia spread radially on the surface of culture media. Formation of aerial hyphae is remarkable and 300 to 500 sclerotia are formed in a plate (9 cm in diameter). Primary conductive hyphae on the surface of culture media are thick, 4.5 to 9.0  $\mu$ m wide, generally bearing clamp connections at widely spaced septa. Secondary and tertiary hyphae are narrower, and lack clamp connections.

Sclerotia are initially tinged with brown and then gradually turn into blackish brown in color. They are globose, and smooth walled, 1 to 2 mm in diameter.

(ii) Growth on potato dextrose agar is far inferior to that on the above mentioned two media. The surface of the agar medium is covered with mycelia, but aerial hyphae and sclerotia are not formed.

### (2) Physiological Properties

Effects of growth temperature and initial pH of the culture media were examined on malt extract agar, oat meal agar and in potato dextrose solution containing 0.1% of yeast extract. The results are as follows:

## (i)

Growing temperature range: 5°–50° C.  
Optimum growing temperature: 15°–30° C.

## (ii)

Growing pH range: 1.5–8.5  
Optimum growing pH: 3.0–6.0

## (3) Formation of Basidiospores

Immediately after isolation, basidiospores were formed on the agar medium containing 50% of water-extract from tomato stems and leaves. The basidia were clavate to obovoid, 7–9×4–5 μm, sterigmata were 2.5–4.0 μm long and basidiospores were obovoid to clavate, apiculate, curved, 4.5–6.5×3.5–4.5 μm.

On the basis of the observations of morphological characteristics, especially formation of sclerotia, clamp connections and basidiospores, and physiological properties, the fungus was identified as *Corticium rolfsii* with reference to "Compendium of Soil Fungi" Academic Press, London (1980) by K. H. Domsch et al., "Gen-shoku Sakumotsu Byogai Zusetsu" third edition, Yokendo, Tokyo (1967) by Kitajima et al., "The Genera of Hyphomycetes from Soil" Williams & Wilkins, Baltimore (1968) by G. L. Barron, and Phytopathology, 51, 107–128 (1961).

The strain was an isolate from a tomato stem, *Corticium rolfsii*, is also called by the name of "KOTSUBU KOYAKUTAKE" or "SHIRAKINU BYOKIN" in Japan and it is one of plant pathogens belonging to Basidiomycetes.

*Pellicularia rolfsii*, *Botryobasidium rolfsii*, *Corticium centrifugum*, and *Athelia rolfsii* are synonyms for *Corticium rolfsii*, and *Sclerotia rolfsii* is the name given to the anamorph of the fungus.

*Corticium rolfsii* IFO 4878 and IFO 6146 have almost same properties with the present strain, but they show the following morphological differences from the strain used in the present invention.

(a) The present strain forms small sclerotia, almost globose in uniform sizes. As compared with this, the sizes of sclerotia formed by IFO strains are dispersed 0.8–3.5 mm, and the shapes are heteromorphic.

(b) The IFO strains form relatively few sclerotia, 10–100 per plate, (0.2–2 sclerotia/cm<sup>2</sup> on average), whereas the present strain forms abundant sclerotia, 300–500 per plate (5–8 sclerotia/cm<sup>2</sup> on average).

From the above mentioned characteristics, the present organism were differentiated from IFO strains and determined as a novel strain. Therefore, we referred to this strain as *Corticium rolfsii* AHU 9627 in order to distinguish the strain from the others and deposited the strain in the Fermentation Research Institute, the Agency of Industrial Science of Technology of Japan, by International Deposit No. 1033 (FERM BP-1033).

In order to obtain a useful amylase (hereinafter referred to as present enzyme) in the present invention, the strain is inoculated in the liquid medium containing nutrients and cultured as usual. After incubation, the enzyme capable of saccharifying raw-starch is collected from culture broth.

The enzyme-producing organism includes not only the AHU 9627 strain, but also all strains belonging to

the same species and their variants capable of producing the present enzyme.

The artificial variants of the AHU 9627 may be readily obtained by UV irradiation, cobalt 60 irradiation or treatments with chemical variation-inducing agents.

These strains and variants can be grown on the culture media for ordinary basidiomycetes, for example, liquid media, solid media and the like. The following carbon sources are useful for the enzyme production: cooked or uncooked starch made from potato, cassave, rice and the like; refined rice bran; soybean flour; corn meal; disaccharides such as maltose, lactose, sucrose and the like; monosaccharides such as glucose, fructose, mannose and the like; and dextrin. Of these carbon sources, uncooked potato starch scarcely accumulated reducing sugar in culture media even at a high concentration of carbon source and it was selected as the most useful carbon source by the fact that the enzyme production was suppressed if the reducing sugar in the medium exceeded 20 mg/ml.

The useful nitrogen sources for the enzyme production are corn steep liquor (CSL), casein, meat extract, peptone, inorganic ammonium salts and the like. Of these substances, peptone was preferred, because it did not produce mucilaginous materials.

In addition to the above nutrients, suitable amounts of inorganic salts such as KH<sub>2</sub>PO<sub>4</sub>, MgSO<sub>4</sub>, FeSO<sub>4</sub>, MnSO<sub>4</sub>, CaCl<sub>2</sub>, CoCl<sub>2</sub>, KCl, NaCl and the like, organic trace elements and surfactants such as Tween 40, Tween 80, Span 80 and the like may be added, if they are necessary.

A satisfactory amount of the enzyme can be produced, when the organism is inoculated in the medium containing the above mentioned nutrients and incubated at a temperature between 15° and 30° C., preferably around 27° C., for 5 to 10 days.

The supernatant and concentrate of the culture broth are usable as the enzyme solution, and the enzyme preparations made from the culture supernatant are also useful for saccharification of raw-starch. The enzyme can be partially or completely purified by the following methods: salting-out with ammonium sulfate, sodium chloride and the like, ion-exchange chromatography, isoelectric precipitation, fractionation with solvent, adsorption chromatography and so forth.

The present enzyme has the following properties.

## (i) Effect of pH

The present enzyme is active between pH 2.0 and 7.0. The optimum pH for the enzyme activity is 4.0 for raw starch, and 4.5 for gelatinized starch. The present enzyme is stable in the pH range from 3.0 to 7.5, especially from 4.0 to 5.0.

## (ii) Effect of temperature

The effect of temperature on the present enzyme activity was examined at pH 4.5 in a solution of 0.05M acetate buffer. The optimum temperature for the present enzyme activity is in the range from 50° to 70° C., especially from 60° to 65° C. In consideration of the enzyme stability, it is in the range from 20° to 60° C., preferably from 40° to 50° C.

## (iii) Action on various kinds of raw-starch

The saccharifying ability of the enzyme was examined on various kinds of starch which were made from rice, wheat, sweet potato, waxy corn, tapioca, and sago, at the concentration of 5% at pH 4.0 and 45° C. As a result, it was found that saccharification took place irrespective of the kinds of starch. The data obtained by high performance liquid chromatography showed that

the major hydrolyzate was glucose. Saccharifying activity toward gelatinized starch to that toward raw-starch was in the ratio between 1.5 and 2.5 at 40° C., and the value changed a little depending on the pH of reaction mixture and the reaction temperature.

The enzymological differences between the present enzyme and the enzyme produced by *Corticium rolfsii* IFO 4878 (hereinafter referred to as IFO 4878 enzyme) were examined and the following results were obtained. A saccharification test of uncooked starch was conducted at a high concentration, in order to clarify the enzymological differences between two enzymes. The culture supernatant of each strains was partially purified by salting-out with 60% ammonium sulfate. Two enzyme solutions obtained were used for saccharification of 30% corn starch suspension at pH 3.0 and 4.5, and 35°, 45° and 55° C., respectively (Table 1). The figures in Table 1 show the saccharification rate(%) of the two enzymes.

TABLE 1

Conditions of Saccharification		Saccharification Rate (%)					
		Present Enzyme			IFO 4878 Enzyme		
Temp. (°C.)	pH	24 hrs.	48 hrs.	72 hrs.	24 hrs.	48 hrs.	72 hrs.
35	3.0	77	83	93	53	53	53
	4.5	73	85	98	64	67	69
45	3.0	87	100	100	28	45	39
	4.5	87	100	100	42	45	49
55	3.0	79	81	81	36	36	35
	4.5	86	86	87	45	45	43

From the results, it was found that the present enzyme has the ability to hydrolyze raw-starch almost completely at 35° and 45° C. irrespective of the pH of reaction mixture and saccharification rate reduced to 90% at 55° C. The saccharification rate of the IFO 4878 enzyme was about 70% at pH 4.5 and 35° C., but the value reduced to 40 and 50% at 45° and 55° C., respectively. The above results show that the present enzyme is a novel enzyme which has powerful saccharifying activity and high stability to heat compared with the IFO 4878 enzyme.

In order to carry out the present invention, the present enzyme is acted on raw starch.

Almost all kinds of starch made from rice, wheat, corn, waxy corn, potato, sweet potato, tapioca, sago and the like, and materials containing these starch would be usable for the practice of the invention.

Starch is mixed with suitable amounts of the enzyme solution, and then saccharified under the optimum conditions. It is desirable to carry out the reaction in a buffer solution within the pH range from 2.0 to 7.0, preferably from 4.0 to 4.5, and the temperature range from 20° to 60° C., preferably from 40° to 50° C. Any buffer solution may be usable, if it can keep a desired pH range. Acetate-, citrate-, phosphate-, McIlvaine-, tris-maleate-buffer solution and so forth may be useful.

The present enzyme is usable in any type of the following preparations: culture broth, extract of the organisms, filtrate of the culture broth, and partially or completely purified enzyme. Of these enzyme preparations, the use of purified enzyme is desirable. In order to determine the optimum amounts of the enzyme for saccharification of raw-starch, it is convenient to measure the activity of the enzyme solution to be used by the following methods.

(1) Saccharifying activity toward raw-starch

The reaction mixture, containing an appropriately diluted enzyme solution and 2.5% raw-nonglutinous rice starch in 0.1M Na-acetate buffer (pH 4.5), was incubated with shaking at 40° C. for 1 hour. After centrifugation, reducing sugar liberated into the supernatant was measured by the DNS method [The Journal of Biological Chemistry 31, 710(1967)]. One unit of the enzyme activity was defined as the amount of enzyme that produced 1  $\mu$ mole of glucose per minute under the above mentioned conditions (IU).

#### (2) Glucoamylase activity

The reaction mixture, containing a diluted enzyme solution and 0.5% soluble starch in 0.05M Na-acetate buffer (pH 4.5), was incubated at 40° C. for 30 minutes. The reducing sugar liberated was assayed by the DNS method and one unit of the activity was defined as the amount of enzyme that liberate 1 mg of glucose in 1 ml of reaction mixture (GU).

It is noted that the activity measured by this method is usable only to make a comparison between the present enzyme and other glucoamylases.

The invented saccharifying method has some advantages that raw-starch suspension can be saccharified even with a high substrate concentration, more than 10%, and that the saccharifying reaction can be carried out at a high temperature under acidic conditions, so that the reaction mixtures can be completely prevented from the contamination of various bacteria. If the enzyme was incubated in combination with alcohol- or organic acid-fermenting microorganisms, the present enzyme will produce alcohols or organic acids very effectively.

The present invention is described by way of examples.

#### EXAMPLES 1

Inocula were prepared by seeding 3 sclerotia from *Corticium rolfsii* AHU-9627 onto 15 ml of potato dextrose agar containing 0.1% of yeast extract in 9 cm Petri-dishes. After 5 days incubation at 27° C., the organisms on 2 plates were put into a cup together with the agar medium and homogenized in 50 ml of sterilized water. For enzyme production, 3 to 5 ml each of the homogenate was inoculated into 100 ml of liquid medium in 500 ml Sakaguchi-flasks. The medium for enzyme production contains in g/liter: polypepton, 30g; ammonium nitrate, 3.0g; magnesium sulfate, 1.8g; and Tween 80, 1.0g. pH of the culture medium was adjusted to 6.0. Each flask was incubated at 27° C for 7 days. Raw nonglutinous rice starch was sterilized with ethylene oxide, and then aseptically added to the flasks containing the autoclaved medium. After incubation, the culture filtrate was concentrated by ultraconcentration and dialysis (44 IU/ml).

One gram of each starch, made from rice, wheat, waxy corn, tapioca, sweet potato, sago, and potato, was weighed in 100 ml Erlenmeyer-flasks and mixed with 10 ml of 0.1M acetate buffer (pH 4.0) and 10 ml of the enzyme solution, and then the mixture was incubated at 45° C. The reducing sugar liberated was measured by the DNS method. As shown in FIG. 1, each starch, made from rice, wheat, waxy corn, tapioca, sweet potato and sago, was completely saccharified after 12 hour incubation. About 50% of suspended potato starch was hydrolyzed after 12 hour incubation and 80% after 24 hour incubation.

## EXAMPLE 2

Eighteen milliliters of a raw-starch suspension in 1M acetate buffer (pH 4.5), which contained 6 g of raw-corn starch, was mixed with 2 ml of the enzyme solution prepared in Example 1 (240 GU/ml) and the mixture was incubated on a shaker at 45° C.

The reducing sugar liberated was determined by the DNS method.

As shown in FIG. 2, 73% of the corn starch was hydrolyzed after 24 hour incubation and 98% after 48 hour incubation. The same examination on the commercial glucoamylase preparations showed that the enzymes produced by *Rhizopus* sp. and *Aspergillus* sp. hydrolyzed 72 and 58% of the corn starch after 48 hour incubation, respectively.

## EXAMPLE 3

Forty grams of raw-sweet potato cut into cylindrical pieces, 1 cm in diameter and 0.5 cm in thickness, were put into a 500 ml flask and mixed with 100 ml of the enzyme solution prepared in Example 1 (22 IU/ml), adjusted pH to 4.0, and then the mixture was incubated at 40° C.

After 48 hour incubation, most of starch in the slices was hydrolyzed and the hard tissues of the slices were changed to spongy pieces. The supernatant of the reac-

tion mixture contained about 9.6 g of glucose per 100 ml.

## EXAMPLE 4

One hundred milliliters of a potato starch suspension in 0.1M acetate buffer (pH 6.0), containing 40 g of dried potato starch (Pharmacopoeia), 0.2% (w/w) per starch of heat resistant  $\alpha$ -amylase, 0.1 g of NaCl and 0.2 g of  $\text{Ca}(\text{OH})_2$ , was heated in boiling water at 95° to 100° C. for 10 minutes, and then autoclaved to prepare a solution of 40% liquefied starch (DE 13.5%).

Fifteen milliliters of the liquefied starch solution and 2 ml of the enzyme solution, prepared in the same manner as in Example 1, in 1M acetate buffer (8 GU/g starch) were mixed and diluted to 20 ml. The mixture was incubated at 45° C. During the course of this experiment, it was found that the liquefied starch was completely hydrolyzed after 48 hour incubation.

What is claimed is:

1. A method for the cooking-free saccharification of starch which comprises using an amylase produced by *Corticium rolfsii* AHU 9627 (International Deposit No. FERM BP-1033) or its variants.

2. A method according to claim 1, wherein a culture filtrate of the *Corticium rolfsii* AHU 9627 (International Deposit No. FERM BP-1033) or its variants is provided as the amylase.

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## Poster Presentation 6-23

**Ethanol Production from Raw Corn Starch by Saccharification with Glucoamylase from *Aspergillus niger* Mutant M 115 and Fermentation with *Saccharomyces cerevisiae***

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This presentation describes the saccharification of raw starch with glucoamylases derived from parental and mutant cultures of *Aspergillus niger*. The saccharification was brought about with commercial amylase preparation and glucoamylase (GA). The mutant-derived GA served better than that from the parental cultures. In the present investigation, efficiency of glucoamylases produced by the wild and aspartate-resistant mutant cultures of *A. niger* has been tested by the processes of pre-saccharification with fermentation (called combined saccharification and fermentation, CSF) and simultaneous saccharification and fermentation (SimSF). The 12.0% solutions of raw cornstarch and soluble starch were saccharified using pre-determined volumes of crude enzyme filtrates in shake flasks at 37°C and 100 rpm. The saccharification was carried out for 24 h and liberated sugars were fermented by adding an inoculum of *Saccharomyces cerevisiae*. Glucose under the same conditions served as a control. Almost 100% of the glucose was consumed whereas residual sugars were present in maize starch and soluble starch hydrolyzates. Low yields in the CSF process occurred due to inhibition of enzymes by maltose/glucose. This process was, however, partially successful as the value obtained for ethanol was 75% of that with simultaneous saccharification and fermentation.

In the simultaneous saccharification and fermentation system, the enzyme-liberated glucose from 12% starch was fermented to ethanol. The maximum yield of ethanol was 57.3 g/L and 47.0 g/L ca 60 g/L produced from glucose;  $Y_{p/s}$  of 0.49 and 0.47 g/g starch while maximum product formation rate of ethanol was 1.12, and 1.03 g/L/h from mutant and parental enzymes. The comparison of kinetic parameters indicated that mutant M115 showed almost all kinetic parameters for product formation and substrate consumption higher than those exhibited by the parental cultures. These values of product formation are remarkably high. The enzyme system produced by the mutant can be applied for successful ethanol production in SimSF.

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**Lewis et al.**

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(54) **METHOD FOR PRODUCING ETHANOL  
USING RAW STARCH**

**Related U.S. Application Data**

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Brandon, SD (US); John Michael  
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Roth, Olivet, SD (US)**

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**Publication Classification**

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**MINNEAPOLIS, MN 55402-0903 (US)**

(57) **ABSTRACT**

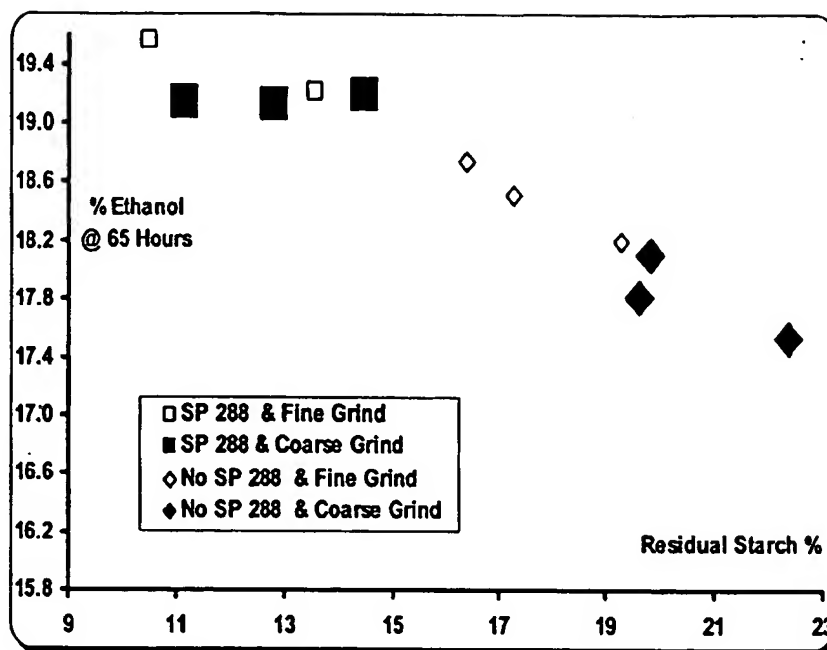
(73) **Assignee: Broin and Associates, Inc., Sioux Falls,  
SD**

The present invention relates to methods for producing high levels of alcohol during fermentation of plant material, and to the high alcohol beer produced. The present invention also relates to methods for producing high protein distiller's dried grain from fermentation of plant material, and to the high protein distiller's dried grain produced. The present invention further relates to reduced stack emissions from drying distillation products from the production of ethanol.

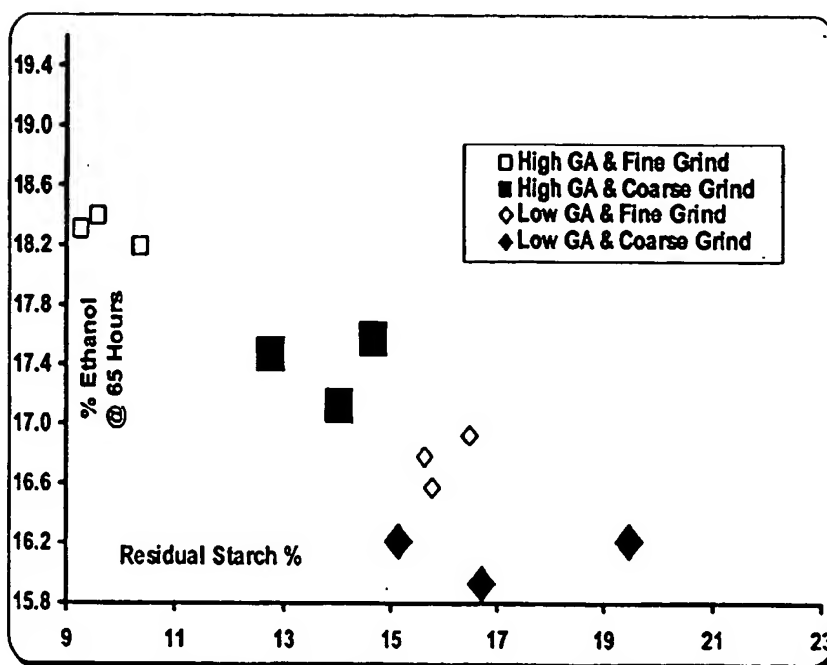
(21) **Appl. No.: 10/798,226**

(22) **Filed: Mar. 10, 2004**

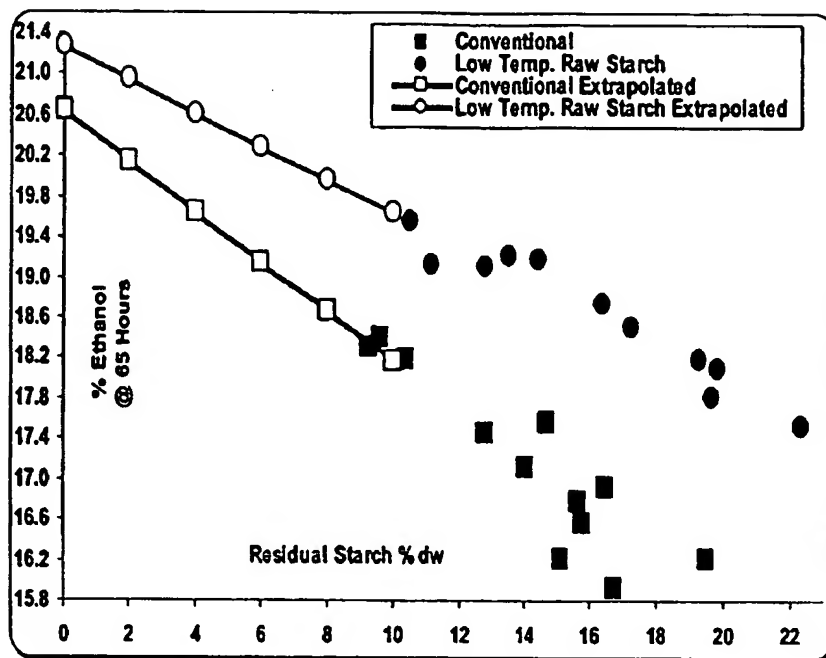
**FIG. 1A**



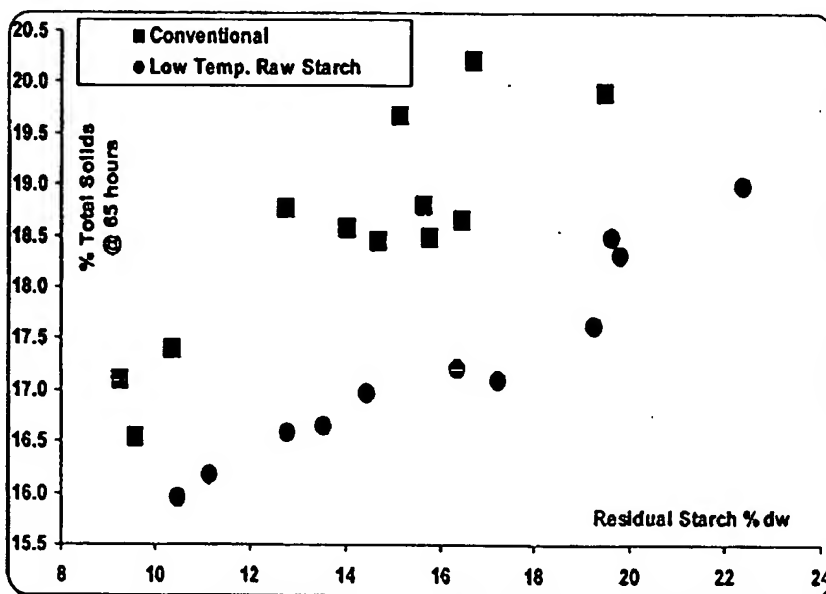
**FIG. 1B**



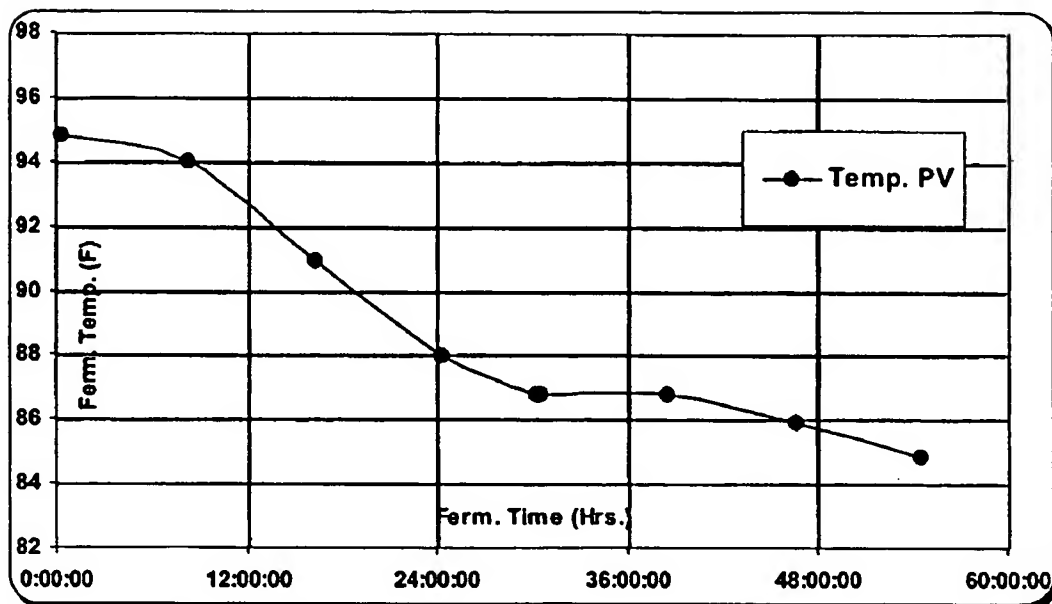
**FIG. 1C**



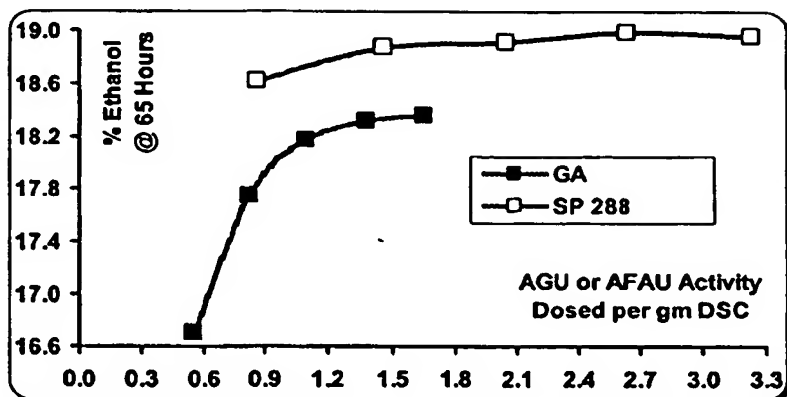
**FIG. 1D**



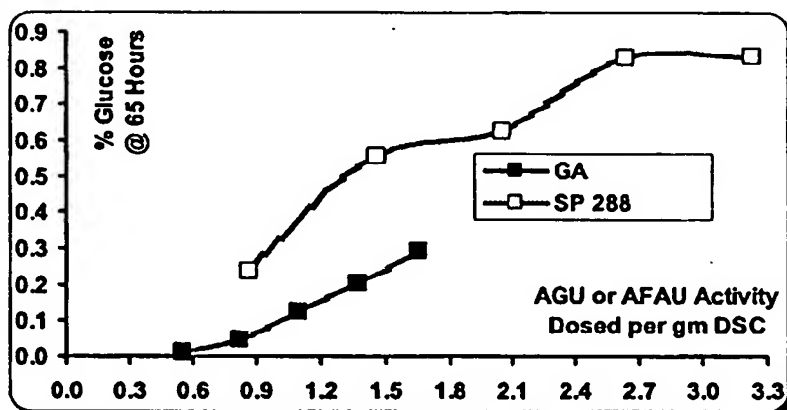
**FIG. 1E**



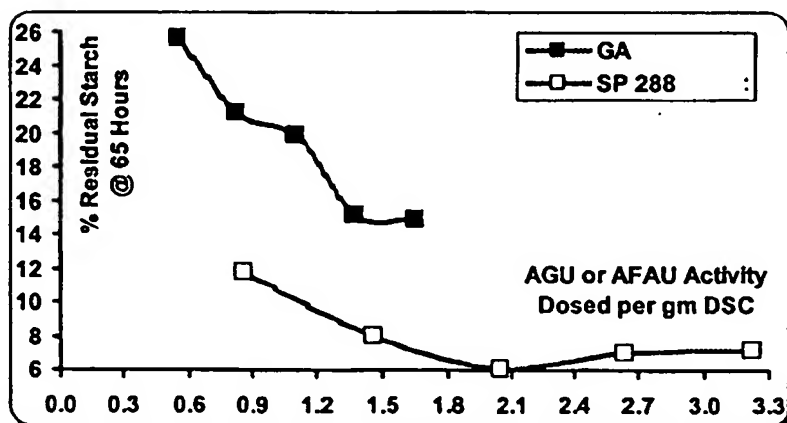
**FIG. 2A**



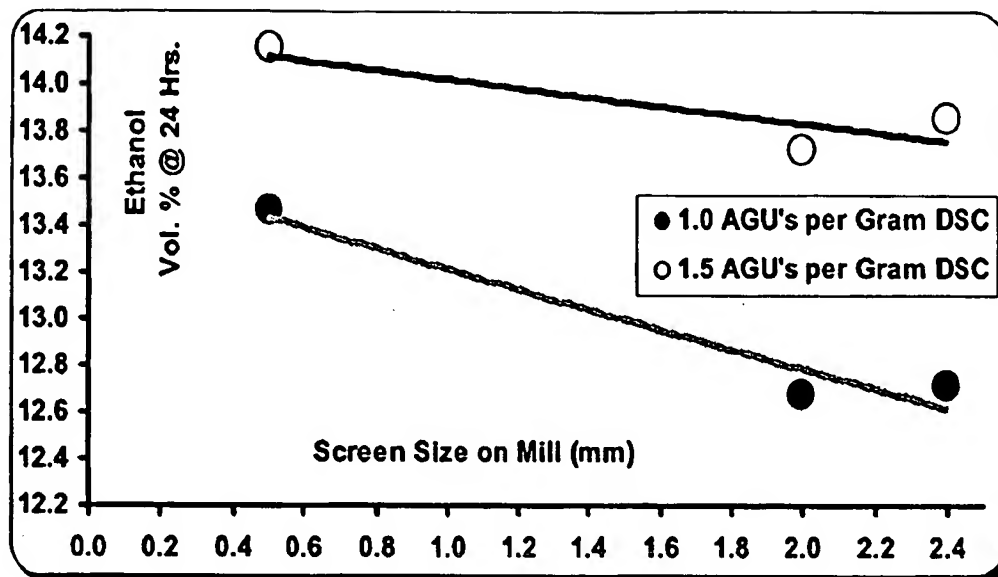
**FIG. 2B**



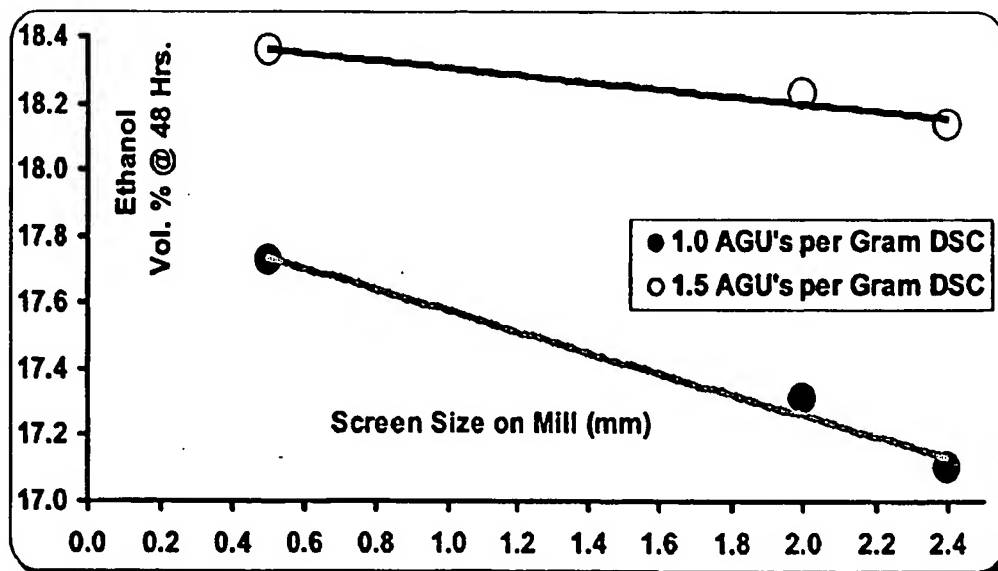
**FIG. 2C**



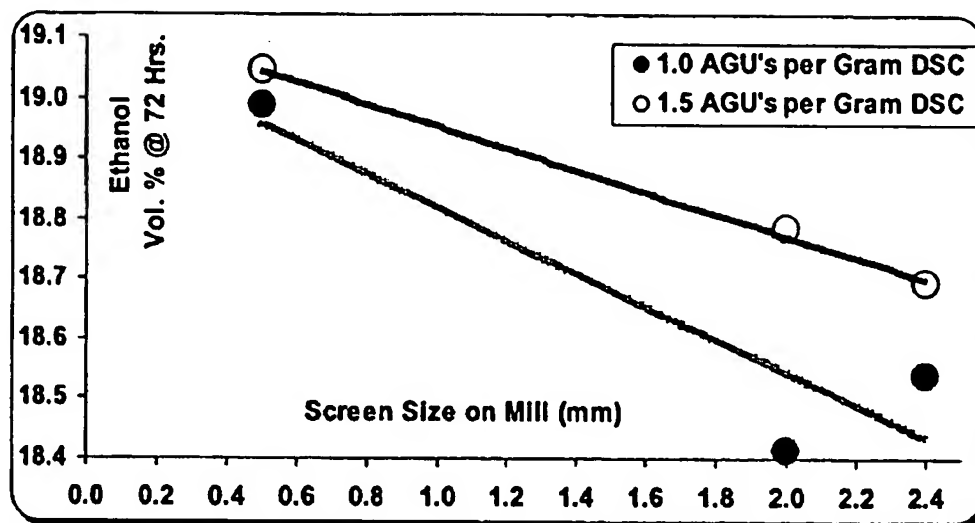
**FIG. 3A**



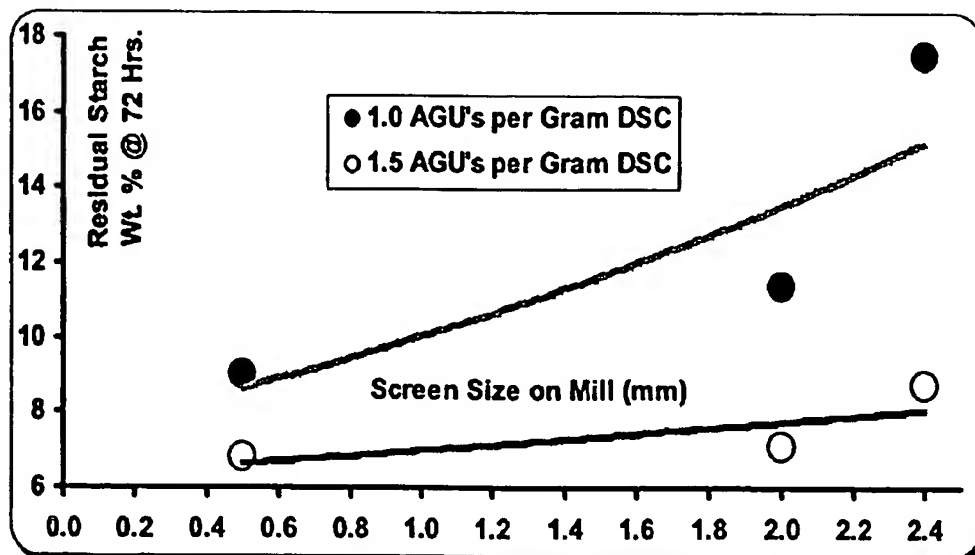
**FIG. 3B**



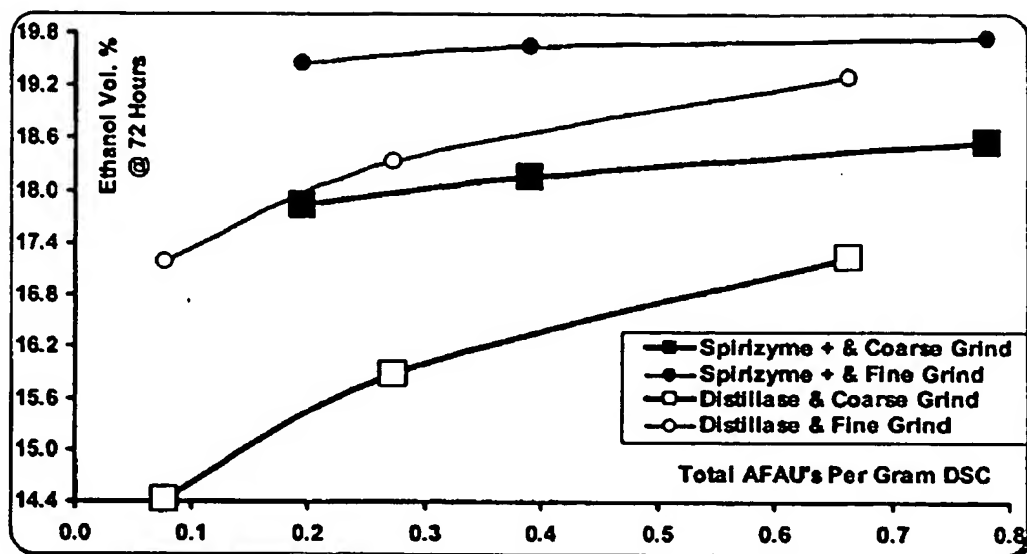
**FIG. 3C**



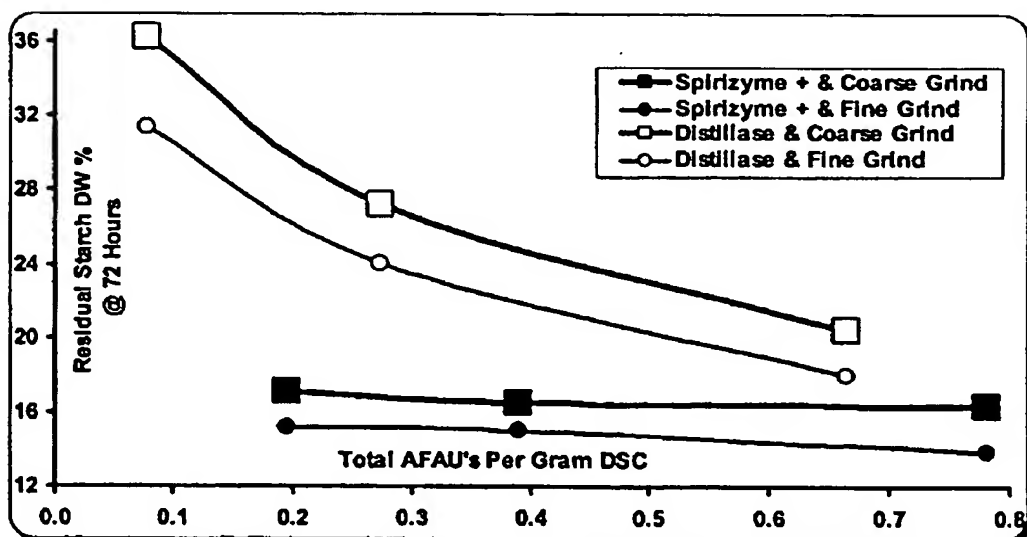
**FIG. 3D**



**FIG. 4A**



**FIG. 4B**



**FIG. 4C**

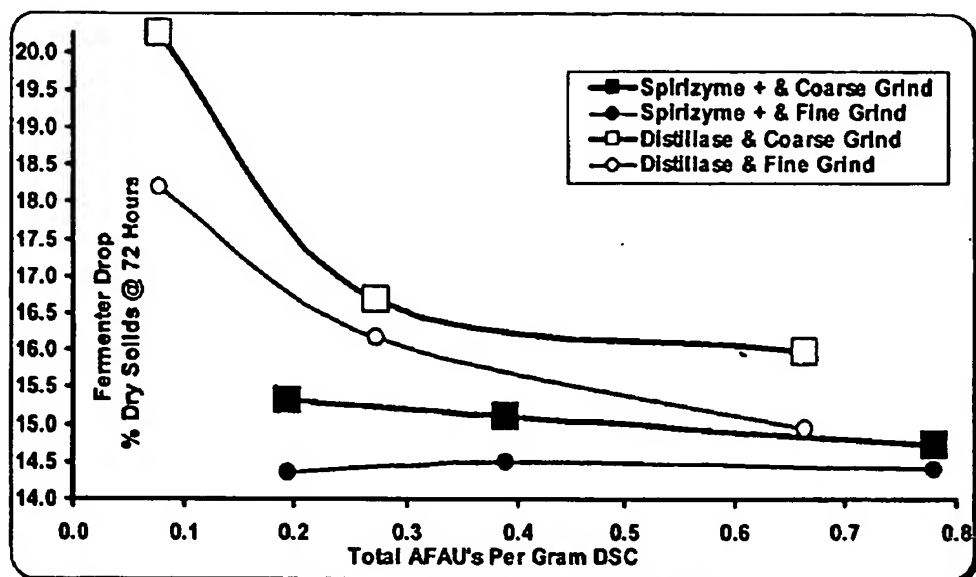


Fig. 5A

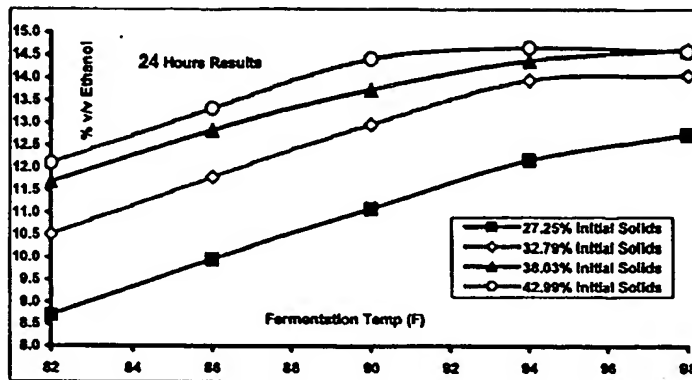


Fig. 5B

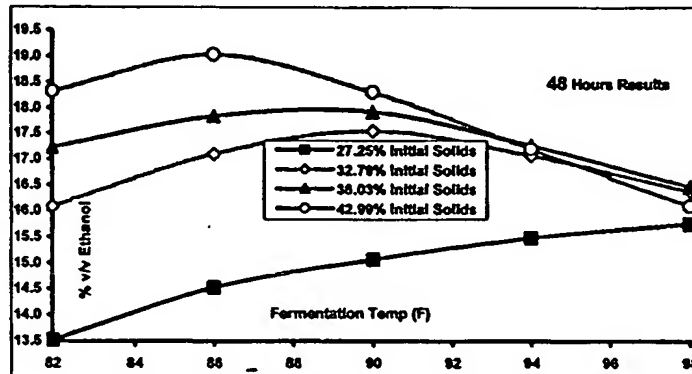


Fig. 5C

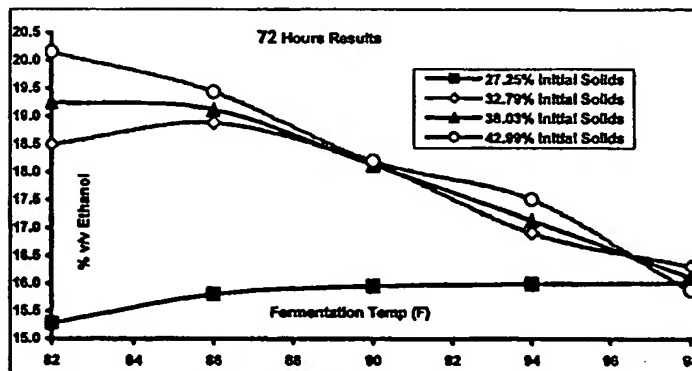
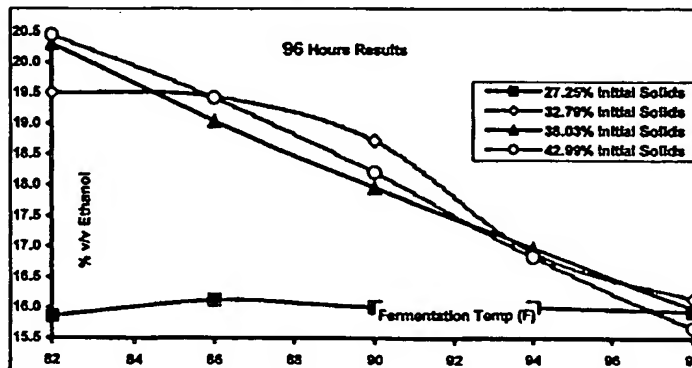


Fig. 5D



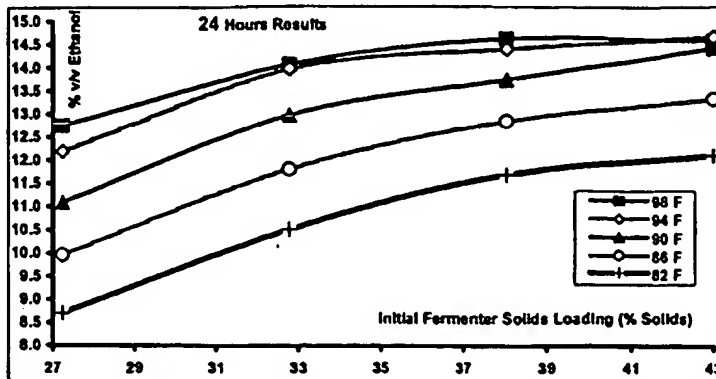


Fig. 5E

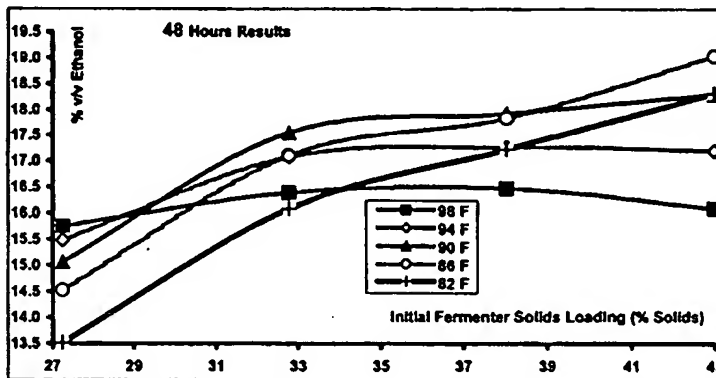


Fig. 5F

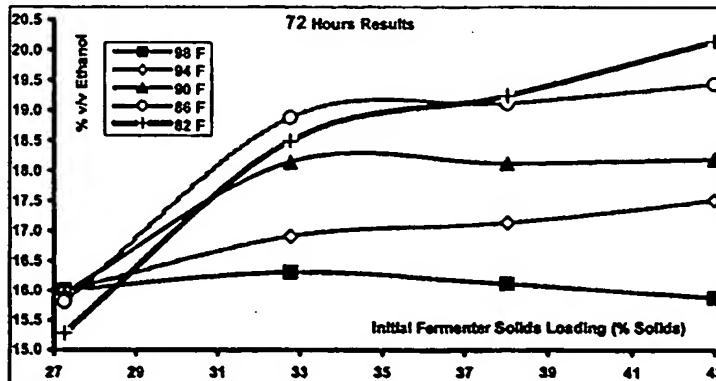


Fig. 5G

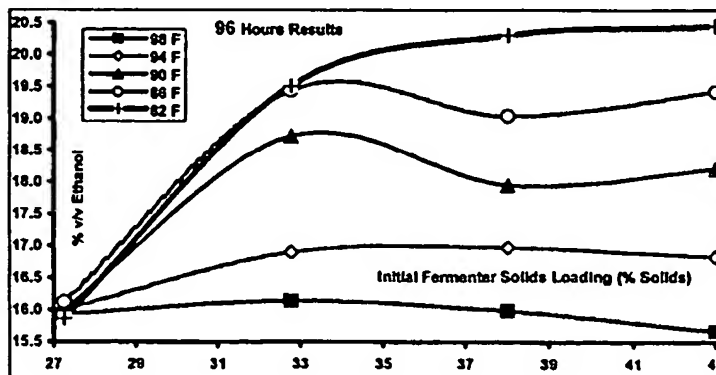


Fig. 5H

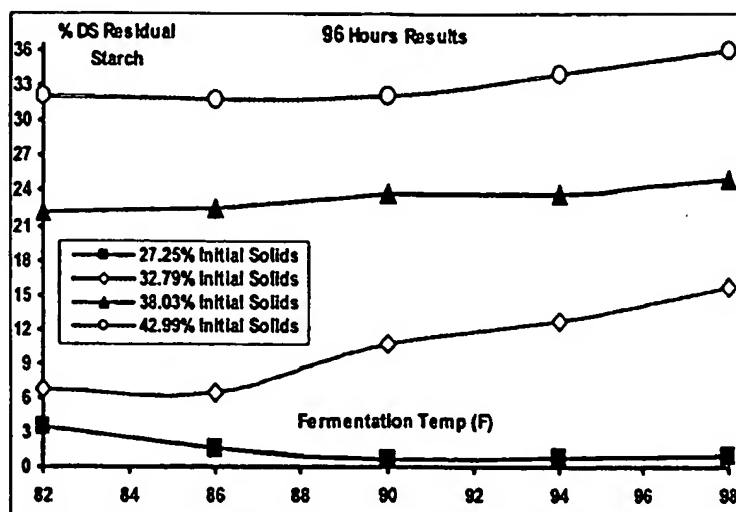


Fig. 5I

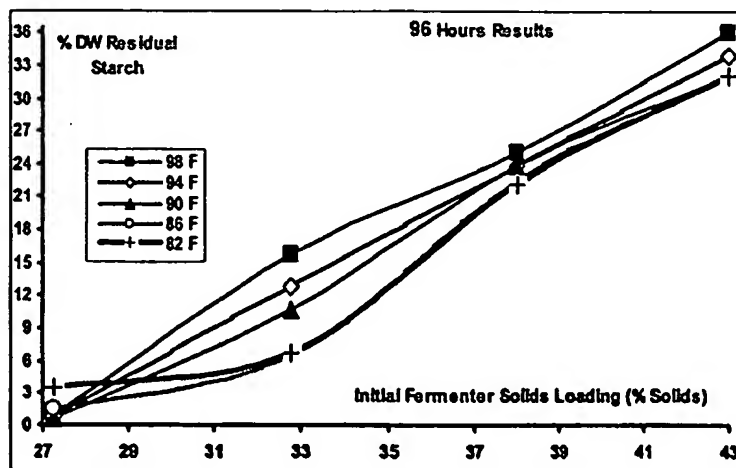
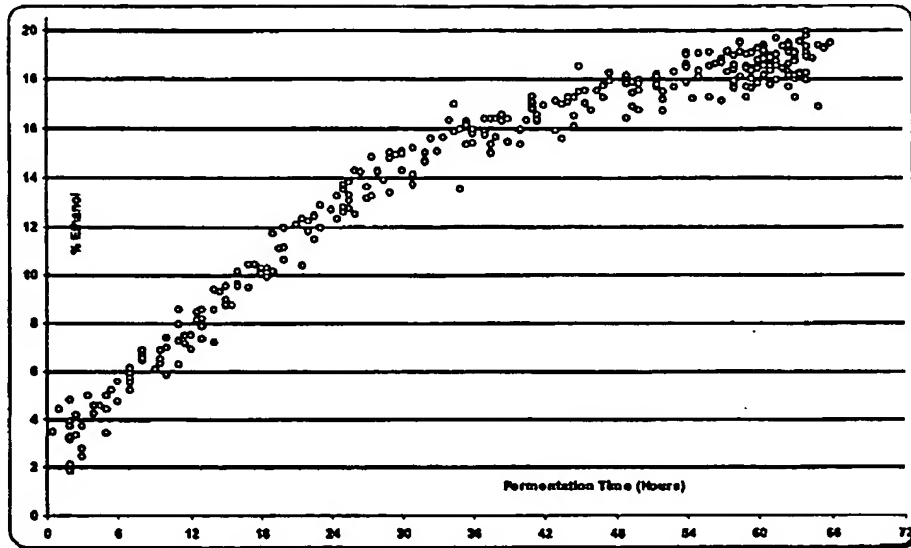


Fig. 5J

**Fig. 6A**



**Fig. 6B**

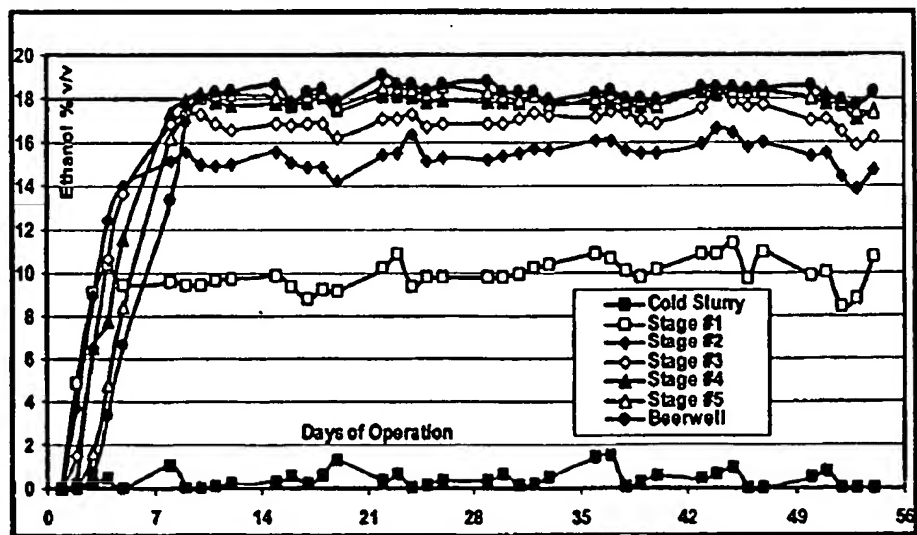
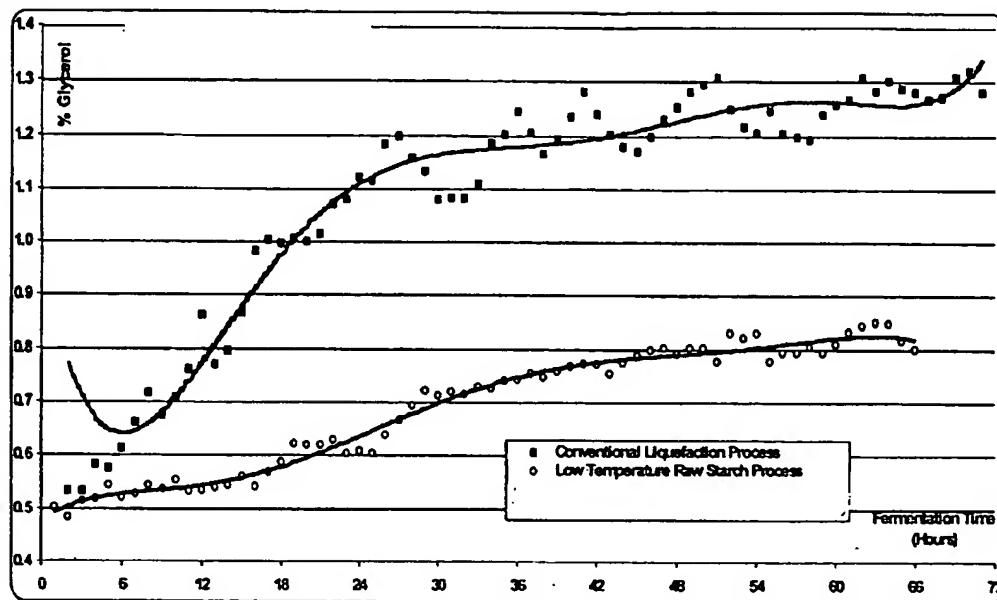


Fig. 7



**Fig. 8**

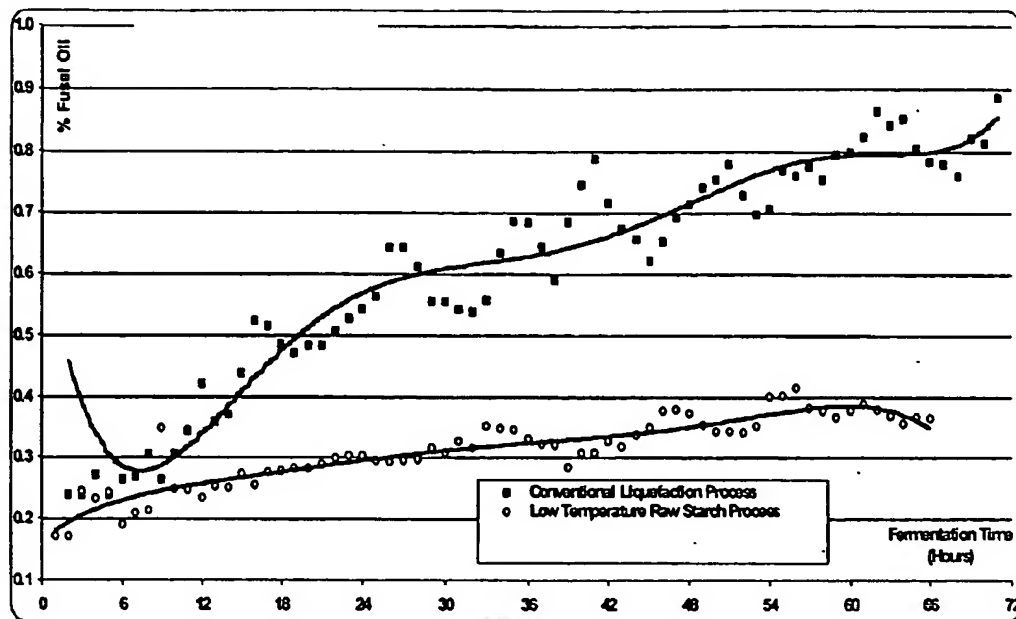


Fig. 9A

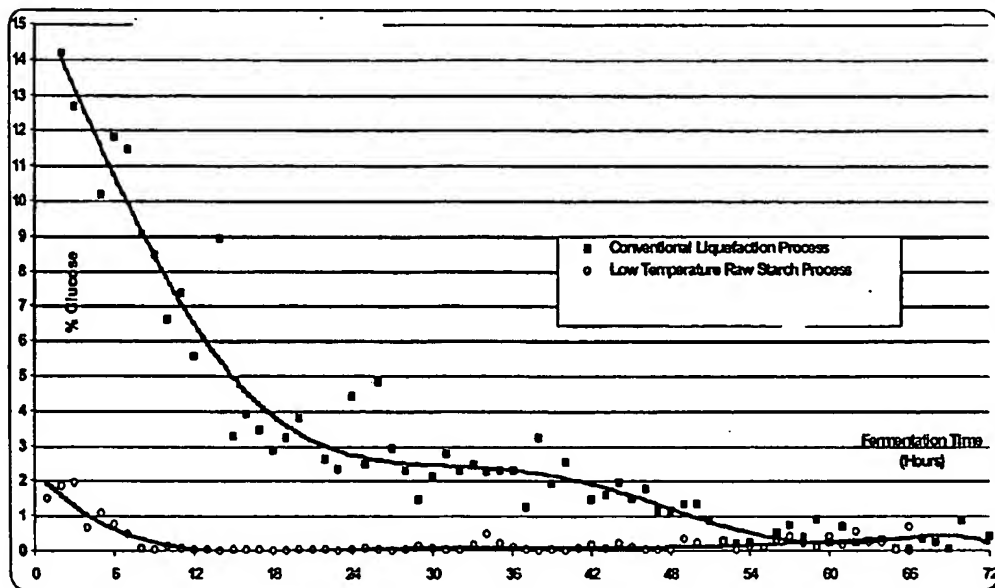
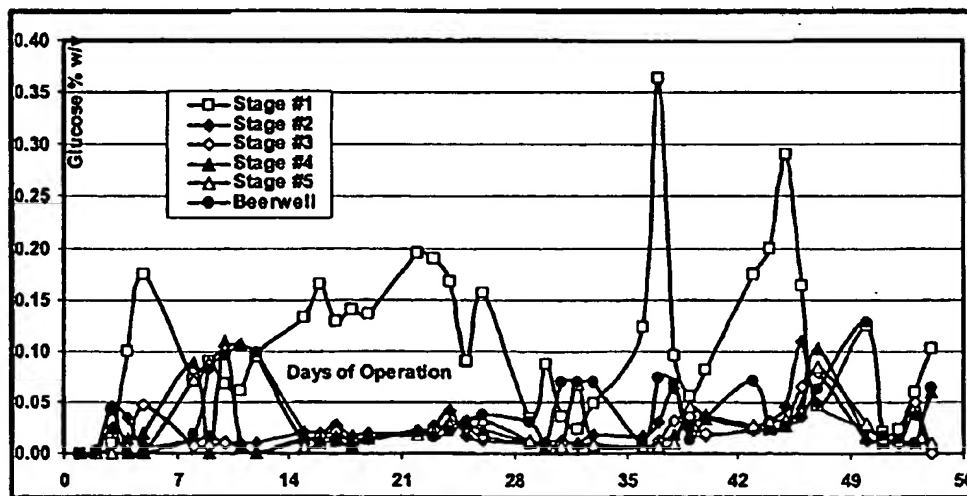
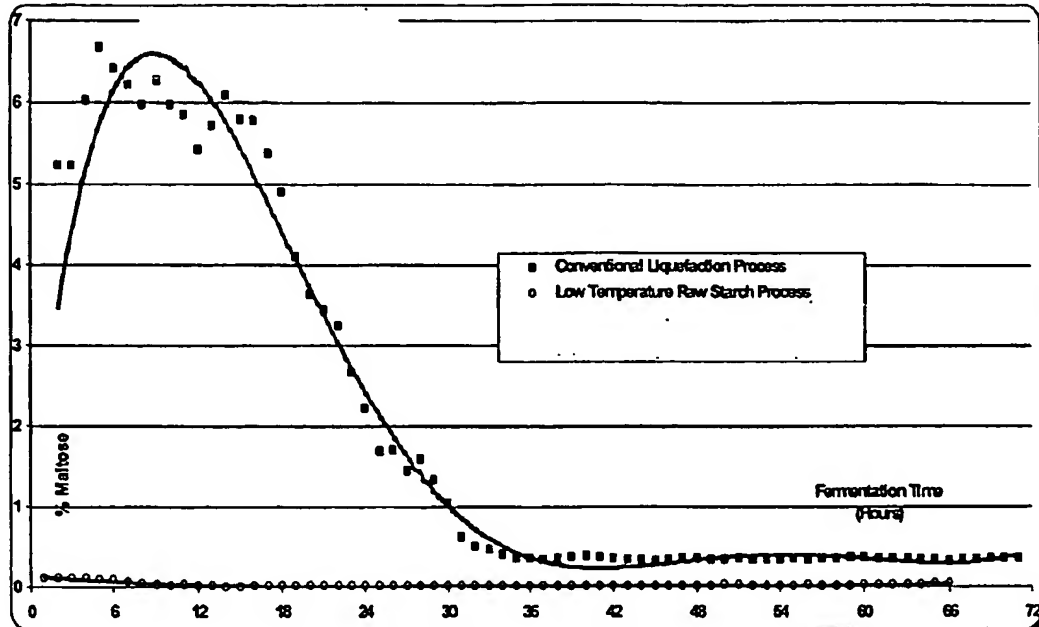


Fig. 9B



**Fig. 10A**



**Fig. 10B**

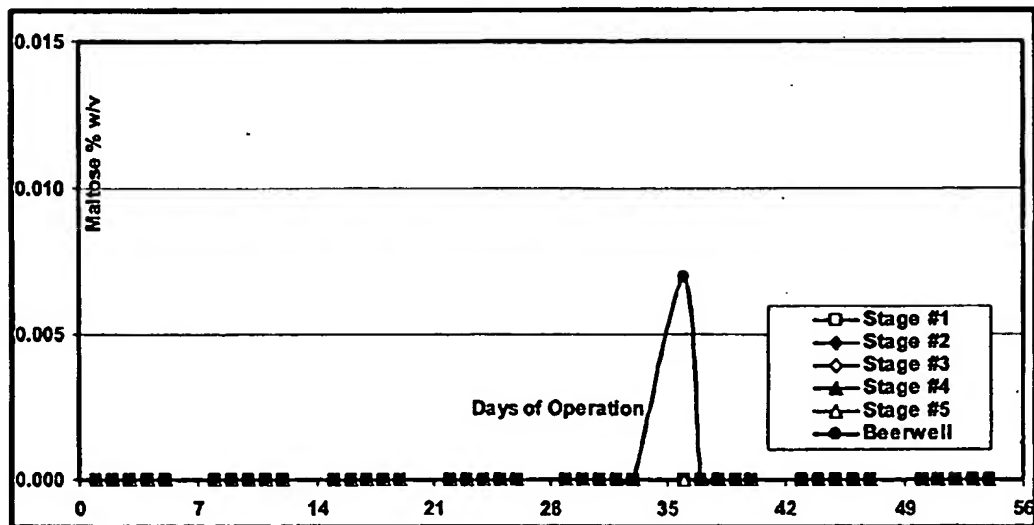


Fig. 11A

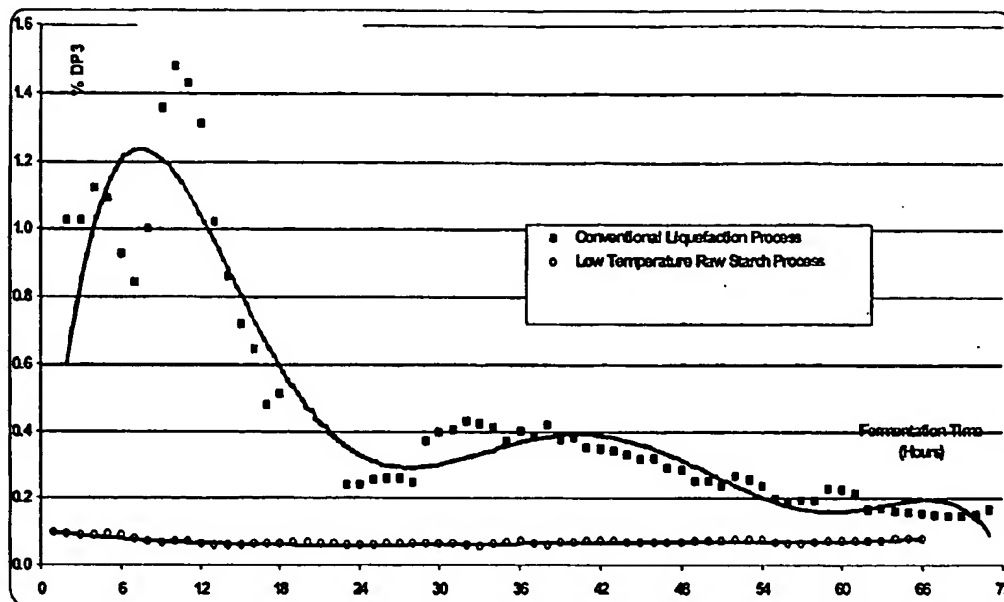


Fig. 11B

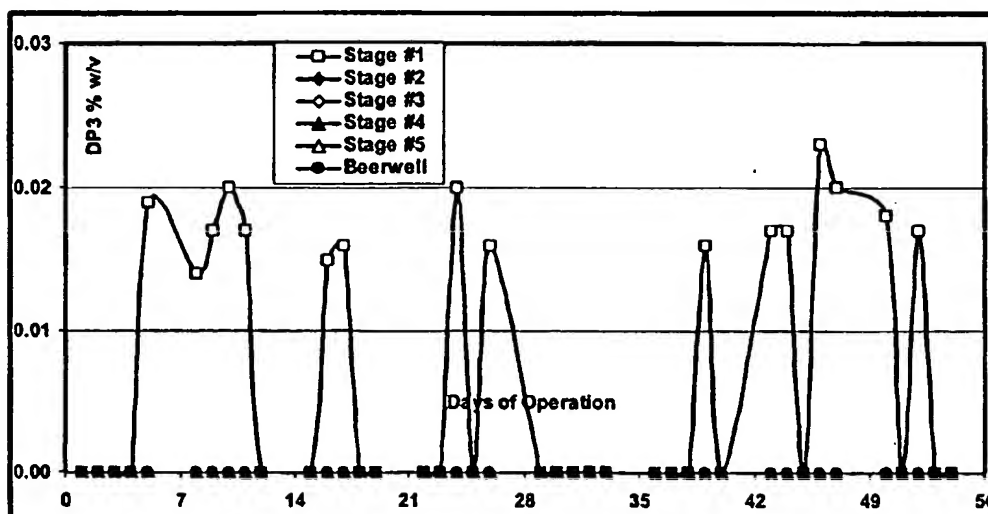


Fig. 12A

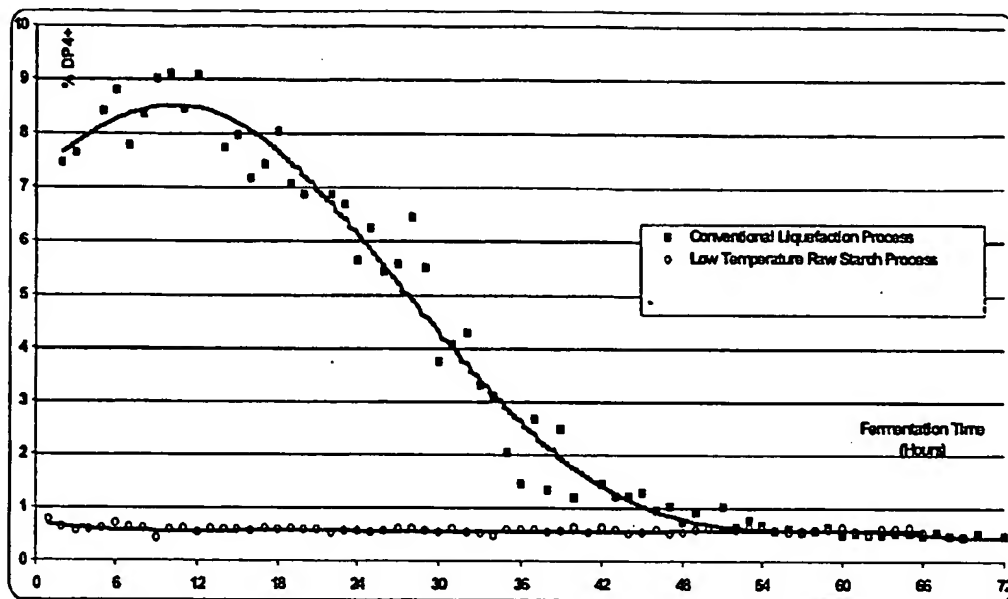


Fig. 12B

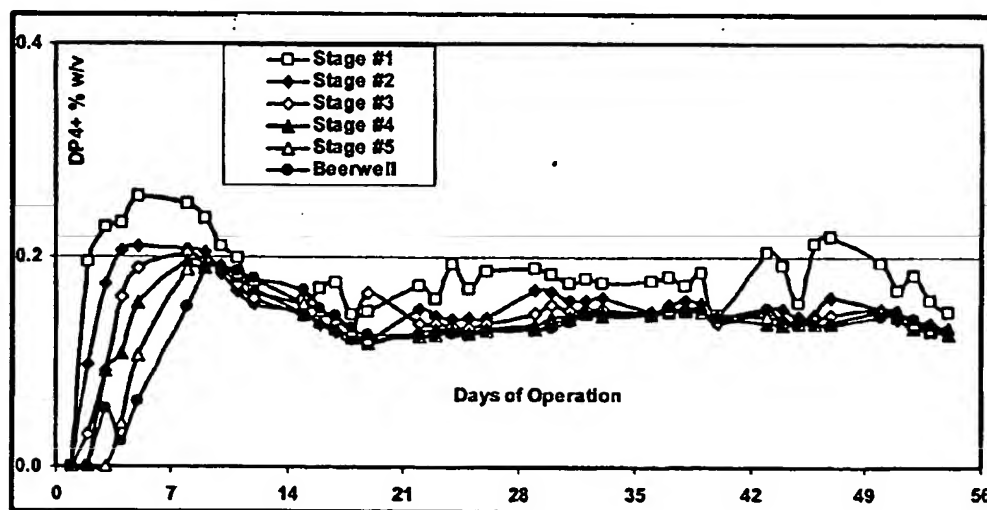
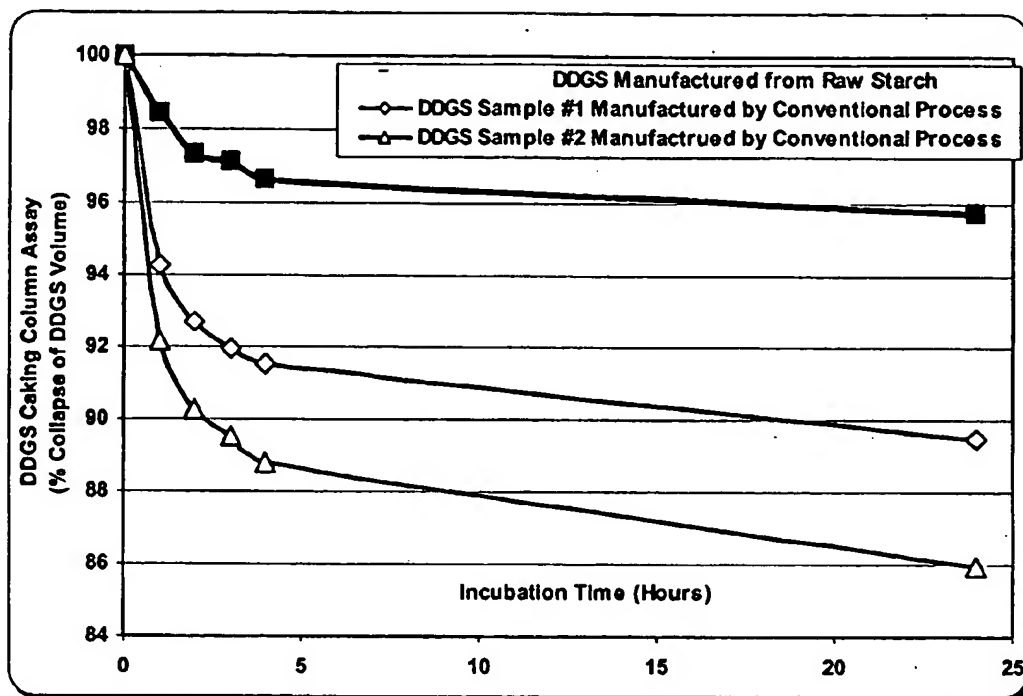


Fig. 13



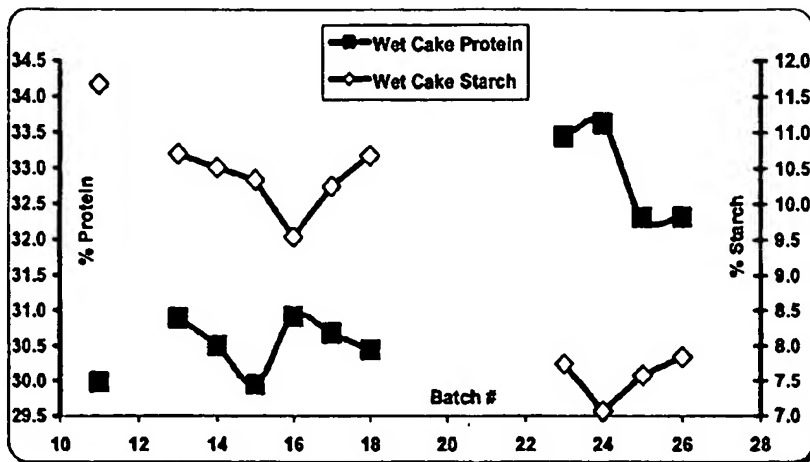


Fig. 14A

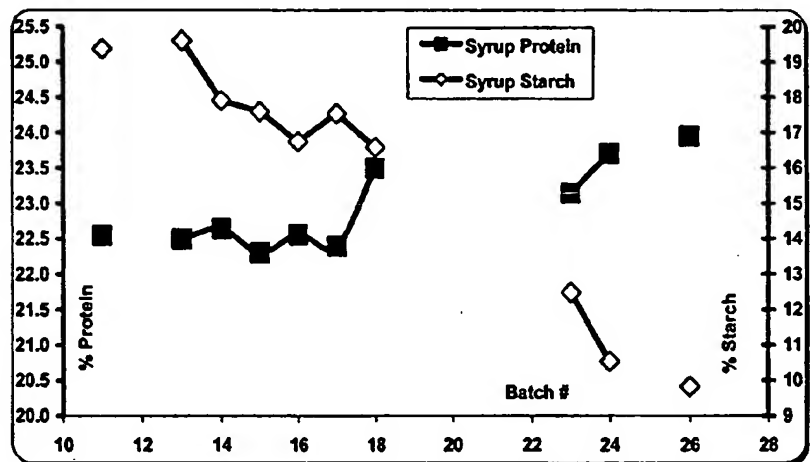
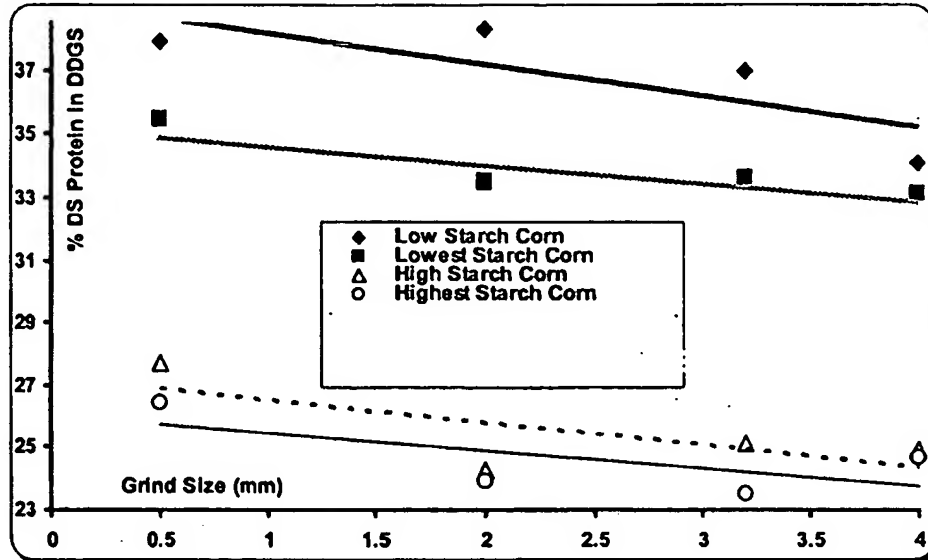
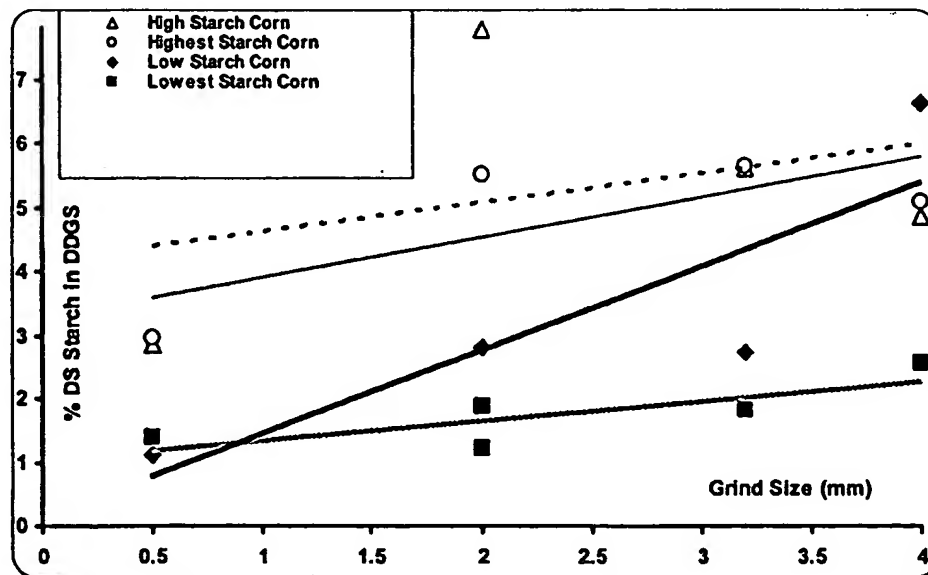


Fig. 14B

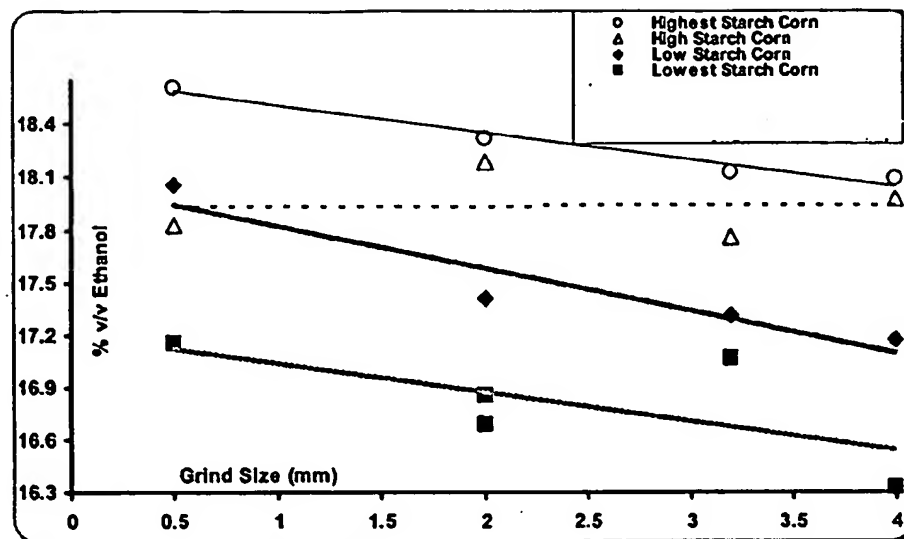
**Fig. 15A**



**Fig. 15B**



**Fig. 15C**



**Fig. 15D**

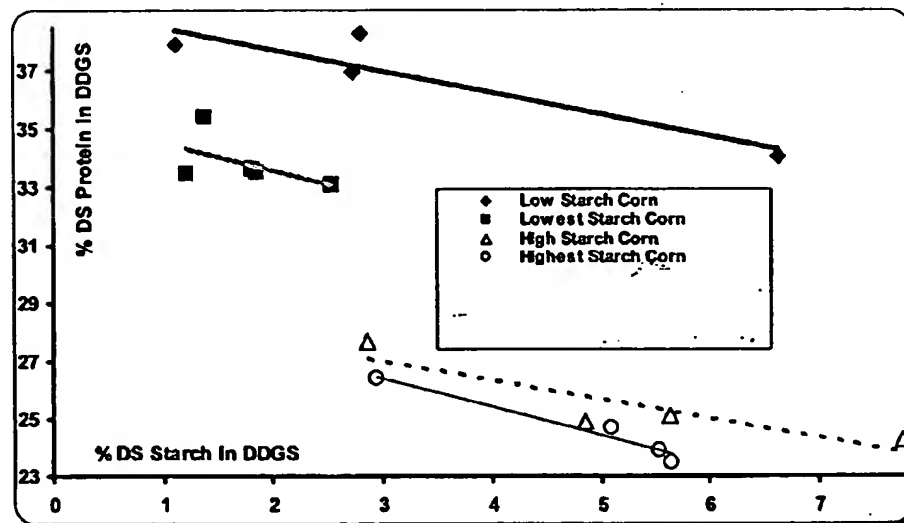


Fig. 16A

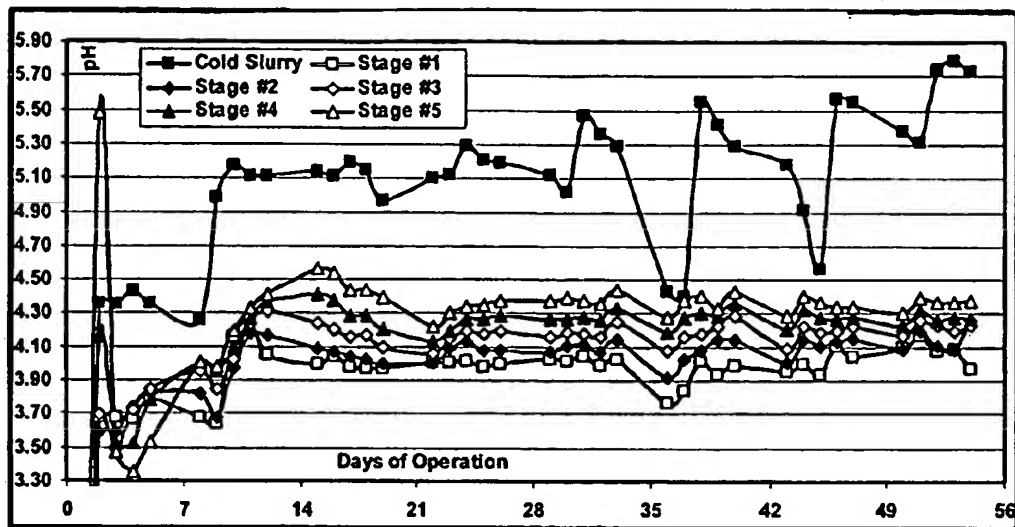


Fig. 16B

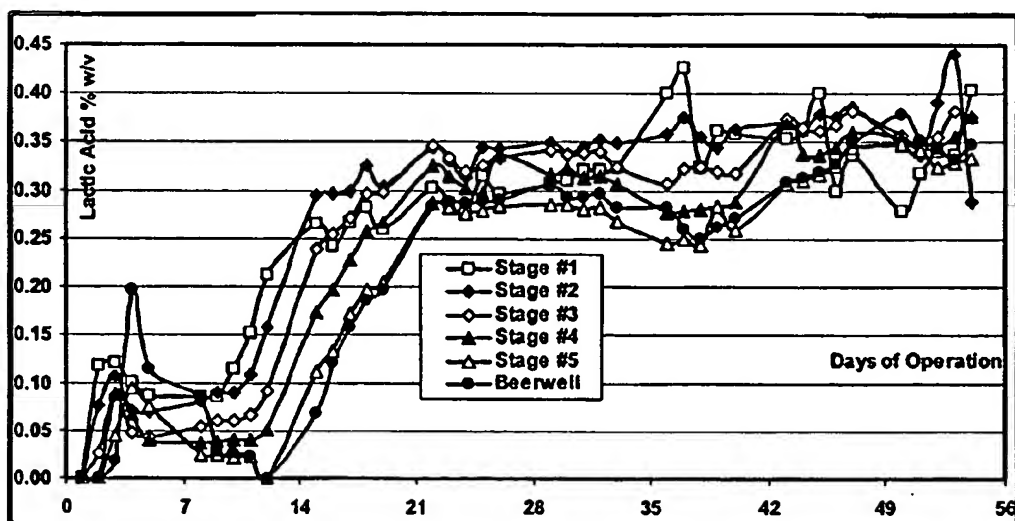
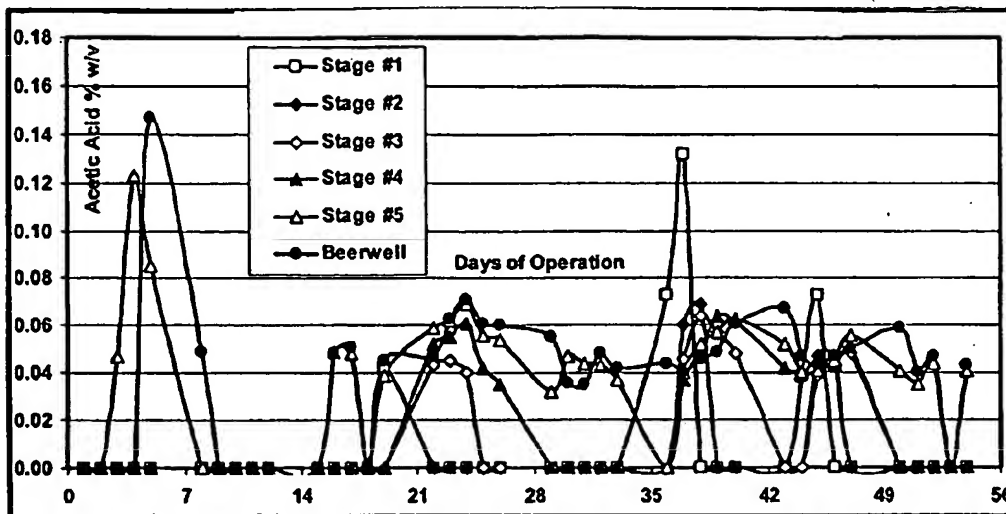


Fig. 16C



**Fig. 17**



## METHOD FOR PRODUCING ETHANOL USING RAW STARCH

### FIELD OF THE INVENTION

[0001] The present invention relates to methods for producing high levels of alcohol during fermentation of plant material, and to the high alcohol beer produced. The present invention also relates to methods for producing high protein distiller's dried grain from fermentation of plant material, and to the high protein distiller's dried grain produced. The present invention further relates to reduced stack emissions from drying distillation products from the production of ethanol.

### BACKGROUND OF THE INVENTION

[0002] Numerous conventional methods exist for converting plant material to ethanol. However, these methods suffer from numerous inefficiencies. There remains a need for additional more effective methods for converting plant material to ethanol and for producing improved fermentation products.

### SUMMARY OF THE INVENTION

[0003] The present invention relates to methods for producing high levels of alcohol during fermentation of plant material, and to the high alcohol beer produced. The present invention also relates to methods for producing high protein distiller's dried grain from fermentation of plant material, and to the high protein distiller's dried grain produced.

[0004] In an embodiment, the present invention relates to a process for producing ethanol from plant material. This method includes grinding the plant material to produce ground plant material including starch; saccharifying the starch, without cooking; fermenting the incubated starch; and recovering the ethanol from the fermentation. The present method can include varying the temperature during fermentation. The present method can include employing a plant material with a particle size such that more than 50% of the material fits through a sieve with a 0.5 mm mesh. The present method can yield a composition including at least 18 vol-% ethanol.

[0005] In an embodiment, the present invention relates to a process for producing high protein distiller's dried grain from plant material. This method includes grinding the plant material to produce ground plant material including starch; producing sugars from the starch without cooking; fermenting the uncooked sugars to yield a composition including ethanol; and recovering distiller's dried grain from the fermentation. The distiller's dried grain can include at least about 30% protein. The distiller's dried grain can include increased levels of the protein zein.

[0006] In an embodiment, the present invention relates to a process of producing ethanol from corn. This process includes producing starch from corn and ethanol from the starch; producing dryer stack emissions including a significantly lower level of volatile organic compounds than conventional technologies.

### BRIEF DESCRIPTION OF THE FIGURES

[0007] FIGS. 1A-E schematically illustrate a comparison of the yield of the process of the present invention compared to the conventional process.

[0008] FIGS. 2A-2C schematically illustrate the effect of dosages of glucoamylase and acid fungal amylase in the present process.

[0009] FIGS. 3A-3D schematically illustrate the effect of grind size and enzyme dosage on fermentation efficiency in the present process.

[0010] FIGS. 4A-4C schematically illustrate the effect of grind particle size, glucoamylase type, and acid fungal amylase dosage on fermentation efficiency in the present process.

[0011] FIGS. 5A-5J schematically illustrate the effect of initial dry solids and temperature on fermentation performance in the present process.

[0012] FIGS. 6A and 6B schematically illustrate high levels of ethanol production from the process of the present invention using simultaneous saccharification and fermentation (SSF) batch or continuous modes of operation.

[0013] FIG. 7 schematically illustrates that the present process maintained low levels of glycerol during SSF batch operations.

[0014] FIG. 8 schematically illustrates that the present process maintained low levels of fusel oils during SSF batch operations.

[0015] FIGS. 9A and 9B schematically illustrate that the present process maintained low levels of glucose during SSF batch or continuous fermentation modes of operation.

[0016] FIGS. 10A and 10B schematically illustrate that the present process maintained low levels of maltose during SSF batch or continuous fermentation modes of operation.

[0017] FIGS. 11A and 11B schematically illustrate that the present process maintained low levels of maltotriose (DP3) during SSF batch or continuous fermentation modes of operation.

[0018] FIGS. 12A and 12B schematically illustrate that the present process maintained low levels of dextrans (DP4+) during SSF batch or continuous fermentation modes of operation.

[0019] FIG. 13 schematically illustrates that the present process impacts DDGS quality favorably based on caking tendency.

[0020] FIGS. 14A and 14B schematically illustrate mass balance of the present process related to proximate separations during the centrifugation step of ethanol production.

[0021] FIGS. 15A-D schematically illustrate that the present process affords advantageous fermentation of non traditional feedstocks.

[0022] FIGS. 16A-C schematically illustrate that the process of the present invention is capable of stable operation in a continuous mode of operation without significant loss due to acid producing bacterial contaminants.

[0023] FIG. 17 schematically illustrates that the present process is capable of achieving low residual starch levels in a continuous mode of operation.

# DETAILED DESCRIPTION OF THE INVENTION

## [0024] Definitions

[0025] As used herein, the phrase "without cooking" refers to a process for converting starch to ethanol without heat treatment for gelatinization and dextrinization of starch using alpha-amylase. Generally, for the process of the present invention, "without cooking" refers to maintaining a temperature below starch gelatinization temperatures, so that saccharification occurs directly from the raw native insoluble starch to soluble glucose while bypassing conventional starch gelatinization conditions. Starch gelatinization temperatures are typically in a range of 57° C. to 93° C. depending on the starch source and polymer type. In the method of the present invention, dextrinization of starch using conventional liquefaction techniques is not necessary for efficient fermentation of the carbohydrate in the grain.

[0026] As used herein, the phrase "plant material" refers to all or part of any plant (e.g., cereal grain), typically a material including starch. Suitable plant material includes grains such as maize (corn, e.g., whole ground corn), sorghum (milo), barley, wheat, rye, rice, and millet; and starchy root crops, tubers, or roots such as sweet potato and cassava. The plant material can be a mixture of such materials and byproducts of such materials, e.g., corn fiber, corn cobs, stover, or other cellulose and hemicellulose containing materials such as wood or plant residues. Suitable plant materials include corn, either standard corn or waxy corn.

[0027] As used herein, the terms "saccharification" and "saccharifying" refer to the process of converting starch to smaller polysaccharides and eventually to monosaccharides, such as glucose. Conventional saccharification uses liquefaction of gelatinized starch to create soluble dextrinized substrate which glucoamylase enzyme hydrolyzes to glucose. In the present method, saccharification refers to converting raw starch to glucose with enzymes, e.g., glucoamylase and acid fungal amylase (AFAU). According to the present method, the raw starch is not subjected to conventional liquefaction and gelatinization to create a conventional dextrinized substrate.

[0028] As used herein, a unit of acid fungal amylase activity (AFAU) refers to the standard Novozymes units for measuring acid fungal amylase activity. The Novozymes units are described in a Novozymes technical bulletin SOP No. EB-SM-0259.02/01. Such units can be measured by detecting products of starch degradation by iodine titration. 1 unit is defined as the amount of enzyme that degrades 5.260 mg starch dry matter per hour under standard conditions.

[0029] As used herein, a unit of glucoamylase activity (GAU) refers to the standard Novozymes units for measuring glucoamylase activity. The Novozymes units and assays for determining glucoamylase activity are described in a publicly available Novozymes technical bulletin.

[0030] As used herein, a unit of amyloglucosidase activity (AGU) refers to the standard Novozymes units for measuring amyloglucosidase activity. The Novozymes units are described in a Novozymes technical bulletin SOP No. EB-SM-0131.02/01. Such units can be measured by detecting conversion of maltose to glucose. The glucose can be determined using the glucose dehydrogenase reaction. 1 unit

is defined as the amount of enzyme that catalyzes the conversion of 1 mmol maltose per minute under the given conditions.

[0031] As used herein, the term "about" modifying any amount refers to the variation in that amount encountered in real world conditions of producing sugars and ethanol, e.g., in the lab, pilot plant, or production facility. For example, an amount of an ingredient employed in a mixture when modified by "about" includes the variation and degree of care typically employed in measuring in an ethanol production plant or lab. For example, the amount of a component of a product when modified by "about" includes the variation between batches in an ethanol production plant or lab and the variation inherent in the analytical method. Whether or not modified by "about," the amounts include equivalents to those amounts. Any quantity stated herein and modified by "about" can also be employed in the present invention as the amount not modified by "about."

## [0032] Converting Starch to Ethanol

[0033] The present invention relates to methods for producing high levels of alcohol during fermentation of plant material, and to the high alcohol beer produced. The present invention also relates to methods for producing high protein distiller's dried grain from fermentation of plant material, to the high protein distiller's dried grain produced, and to the cleaner dryer stack emissions.

[0034] The present method converts starch from plant material to ethanol. In an embodiment, the present method can include preparing the plant material for saccharification, converting the prepared plant material to sugars without cooking, and fermenting the sugars.

[0035] The plant material can be prepared for saccharification by any a variety of methods, e.g., by grinding, to make the starch available for saccharification and fermentation. In an embodiment, the vegetable material can be ground so that a substantial portion, e.g., a majority, of the ground material fits through a sieve with a 0.1-0.5 mm screen. For example, in an embodiment, about 70% or more, of the ground vegetable material can fit through a sieve with a 0.1-0.5 mm screen. In an embodiment, the reduced plant material can be mixed with liquid at about 20 to about 50 wt-% or about 25 to about 45 wt-% dry reduced plant material.

[0036] The present process can include converting reduced plant material to sugars that can be fermented by a microorganism such as yeast. This conversion can be effected by saccharifying the reduced plant material with an enzyme preparation, such as a saccharifying enzyme composition. A saccharifying enzyme composition can include any of a variety of known enzymes suitable for converting reduced plant material to fermentable sugars, such as amylases (e.g.,  $\alpha$ -amylase and/or glucoamylase). In an embodiment, saccharification is conducted at a pH of about 6.0 or less, for example, about 4.5 to about 5.0.

[0037] The present process includes fermenting sugars from reduced plant material to ethanol. Fermenting can be effected by a microorganism, such as yeast. In an embodiment, fermentation is conducted at a pH of about 6 or less, for example, about 4.5 to about 5. In an embodiment, the present method can include varying the pH. For example, fermentation can include filling the fermenter at pH of about

3 to about 4.5 during the first half of fill and at a pH of about 4.5 to about 6 during the second half of the fermenter fill cycle. In an embodiment, fermentation is conducted at a temperature of about 25 to about 40° C. or about to about 35° C. In an embodiment, during fermentation the temperature is decreased from about 40° C. to about 30° C. or about 25° C., or from about 35° C. to about 30° C., during the first half of the fermentation, and the temperature is held at the lower temperature for the second half of the fermentation. In an embodiment, fermentation is conducted for about to (e.g., 24) to about 150 hours, for example, for about 48 (e.g., 47) to about 96 hours.

[0038] The present process can include simultaneously converting reduced plant material to sugars and fermenting those sugars with a microorganism such as yeast.

[0039] The product of the fermentation process is referred to herein as "beer". Ethanol can be recovered from the fermentation mixture, from the beer, by any of a variety of known processes, such as by distilling. The remaining stillage includes both liquid and solid material. The liquid and solid can be separated by, for example, centrifugation.

[0040] Preparing the Plant Material

[0041] The present method converts starch from plant material to ethanol. The plant material can be reduced by a variety of methods, e.g., by grinding, to make the starch available for saccharification and fermentation. Other methods of plant material reduction are available. For example, vegetable material, such as kernels of corn, can be ground with a ball mill, a roller mill, a hammer mill, or another mill known for grinding vegetable material, and/or other materials for the purposes of particle size reduction. The use of emulsion technology, rotary pulsation, and other means of particle size reduction can be employed to increase surface area of plant material while raising the effectiveness of flowing the liquefied media. The prepared plant material can be referred to as being or including "raw starch".

[0042] A fine grind exposes more surface area of the plant material, or vegetable material, and can facilitate saccharification and fermentation. In an embodiment, the vegetable material is ground so that a substantial portion, e.g., a majority, of the ground material fits through a sieve with a 0.1-0.5 mm screen. In an embodiment, about 35% or more of the ground vegetable material can fit through a sieve with a 0.1-0.5 mm screen. In an embodiment, about 35 to about 70% of the ground vegetable material can fit through a sieve with a 0.1-0.5 mm screen. In an embodiment, about 50% or more of the ground vegetable material can fit through a sieve with a 0.1-0.5 mm screen. In an embodiment, about 90% of the ground vegetable material can fit through a sieve with a 0.1-0.5 mm screen. In an embodiment, all of the ground vegetable material can fit through a sieve with a 0.1-0.5 mm screen.

[0043] Fractionation

[0044] In an embodiment, the vegetable material can be fractionated into one or more components. For example, a vegetable material such as a cereal grain or corn can be fractionated into components such as fiber (e.g., corn fiber), germ (e.g., corn germ), and a mixture of starch and protein (e.g., a mixture of corn starch and corn protein). One or a mixture of these components can be fermented in a process according to the present invention. Fractionation of corn or

another plant material can be accomplished by any of a variety of methods or apparatus. For example, a system manufactured by Satake can be used to fractionate plant material such as corn.

[0045] Saccharification and Fermentation

[0046] Saccharification

[0047] The present process can include converting reduced plant material to sugars that can be fermented by a microorganism such as yeast. This conversion can be effected by saccharifying the reduced plant material with any of a variety of known saccharifying enzyme compositions. In an embodiment, the saccharifying enzyme composition includes an amylase, such as an alpha amylase (e.g., acid fungal amylase). The enzyme preparation can also include glucoamylase. The enzyme preparation need not, and, in an embodiment, does not include protease. However, ethanol production methods according to the present invention can conserve water by reusing process waters (backset) which may contain protease. In an embodiment, the present method employs acid fungal amylase for hydrolyzing raw starch.

[0048] Saccharifying can be conducted without cooking. For example, saccharifying can be conducted by mixing source of saccharifying enzyme composition (e.g., commercial enzyme), yeast, and fermentation ingredients with ground grain and process waters without cooking.

[0049] In an embodiment, saccharifying can include mixing the reduced plant material with a liquid, which can form a slurry or suspension and adding saccharifying enzyme composition (e.g., at least one of acid fungal amylase and glucoamylase) to the liquid. In an embodiment, the method includes mixing the reduced plant material and liquid and then adding the saccharifying enzyme composition (e.g., at least one of acid fungal amylase and glucoamylase). Alternatively, adding enzyme composition can precede or occur simultaneously with mixing.

[0050] In an embodiment, the reduced plant material can be mixed with liquid at about 20 to about 50 wt-%, about 25 to about 45 (e.g., 44) wt-%, about 30 to about 40 (e.g., 39) wt-%, or about 35 wt-% dry reduced plant material. As used herein, wt-% of reduced plant material in a liquid refers to the percentage of dry substance reduced plant material or dry solids. In an embodiment, the method of the present invention can convert raw or native starch (e.g., in dry reduced plant material) to ethanol at a faster rate at higher dry solids levels compared to conventional saccharification with cooking. Although not limiting to the present invention, it is believed that the present method can be practiced at higher dry solids levels because, unlike the conventional process, it does not include gelatinization, which increases viscosity.

[0051] Suitable liquids include water and a mixture of water and process waters, such as stillage (backset), scrubber water, evaporator condensate or distillate, side stripper water from distillation, or other ethanol plant process waters. In an embodiment, the liquid includes water. In an embodiment, the liquid includes water in a mixture with about 1 to about 70 vol-% stillage, about 15 to about 60 vol-% stillage, about 30 to about 50 vol-% stillage, or about 40 vol-% stillage.

[0052] In the conventional process employing gelatinization and liquefaction, stillage provides nutrients for efficient yeast fermentation, especially free amino nitrogen (FAN) required by yeast. The present invention can provide effective fermentation with reduced levels of stillage and even without added stillage. In an embodiment, the present method employs a preparation of plant material that supplies sufficient quantity and quality of nitrogen for efficient fermentation under high gravity conditions (e.g., in the presence of high levels of reduced plant material). Thus, in an embodiment, no or only low levels of stillage can suffice.

[0053] However, the present method provides the flexibility to employ high levels of stillage if desired. The present method does not employ conventional liquefaction. Conventional liquefaction increases viscosity of the fermentation mixture and the resulting stillage. The present method produces lower viscosity stillage. Therefore, in an embodiment, increased levels of stillage can be employed in the present method without detrimental increases in viscosity of the fermentation mixture or resulting stillage.

[0054] Further, although not limiting to the present invention, it is believed that conventional saccharification and fermentation processes require added FAN due to undesirable "Maillard Reactions" which occur during high temperature gelatinization and liquefaction. The Maillard Reactions consume FAN during cooking. As a result, the conventional process requires adding stillage to increase levels of FAN in fermentation. It is believed that the present process avoids temperature induced Maillard Reactions and provides increased levels of FAN in the reduced plant material, which are effectively utilized by the yeast in fermentation.

[0055] Saccharification can employ any of a variety of known enzyme sources (e.g., a microorganism) or compositions to produce fermentable sugars from the reduced plant material. In an embodiment, the saccharifying enzyme composition includes an amylase, such as an alpha amylase (e.g., acid fungal amylase) or a glucoamylase.

[0056] In an embodiment, saccharification is conducted at a pH of about 6.0 or less, pH of about 3.0 to about 6.0, about 3.5 to about 6.0, about 4.0 to about 5.0, about 4.0 to about 4.5, or about 4.5 to about 5.0. The initial pH of the saccharification mixture can be adjusted by addition of, for example, ammonia, sulfuric acid, phosphoric acid, process waters (e.g., stillage (backset), evaporator condensate (distillate), side stripper bottoms, and the like), and the like. Activity of certain saccharifying enzyme compositions (e.g., at least one of acid fungal amylase and glucoamylase) can be enhanced at pH lower than the above ranges.

[0057] In an embodiment, saccharification is conducted at a temperature of about 25 to about 40° C. or about 30 to about 35° C.

[0058] In an embodiment, saccharifying can be carried out employing quantities of saccharifying enzyme composition (e.g., at least one of acid fungal amylase and glucoamylase) selected to maintain low concentrations of dextrin in the fermentation broth. For example, the present process can employ quantities of saccharifying enzyme composition (e.g., at least one of acid fungal amylase and glucoamylase) selected to maintain maltotriose (DP3) at levels at or below about 0.2 wt-% or at or below about 0.1 wt-%. For example,

the present process can employ quantities of saccharifying enzyme composition (e.g., at least one of acid fungal amylase and glucoamylase) selected to maintain dextrin with a degree of polymerization of 4 or more (DP4+) at levels at or below about 1 wt-% or at or below about 0.5 wt-%. For maintaining low levels of maltotriose and/or DP4+, suitable levels of acid fungal amylase and glucoamylase include about 0.3 to about 3 AFAU/gram dry solids reduced plant material (e.g., DSC) of acid fungal amylase and about 1 to about 2.5 (e.g., 2.4) AGU per gram dry solids reduced plant material (e.g., DSC) of glucoamylase. In an embodiment, the reaction mixture includes about 1 to about 2 AFAU/gram dry solids reduced plant material (e.g., DSC) of acid fungal amylase and about 1 to about 1.5 AGU per gram dry solids reduced plant material (e.g., DSC) of glucoamylase.

[0059] In an embodiment, saccharifying can be carried out employing quantities of saccharifying enzyme composition (e.g., at least one of acid fungal amylase and glucoamylase) selected to maintain low concentrations of maltose in the fermentation broth. For example, the present process can employ quantities of saccharifying enzyme composition (e.g., at least one of acid fungal amylase and glucoamylase) selected to maintain maltose at levels at or below about 0.3 wt-%. For maintaining low levels of maltose, suitable levels of acid fungal amylase and glucoamylase include about 0.3 to about 3 AFAU/gram dry solids reduced plant material (e.g., DSC) of acid fungal amylase and about 1 to about 2.5 (e.g., 2.4) AGU per gram dry solids reduced plant material (e.g., DSC) of glucoamylase. In an embodiment, the reaction mixture includes about 1 to about 2 AFAU/gram dry solids reduced plant material (e.g., DSC) of acid fungal amylase and about 1 to about 1.5 AGU per gram dry solids reduced plant material (e.g., DSC) of glucoamylase.

#### [0060] Acid Fungal Amylase

[0061] In certain embodiments, the present method employs an  $\alpha$ -amylase. The  $\alpha$ -amylase can be one produced by fungi. The  $\alpha$ -amylase can be one characterized by its ability to hydrolyze carbohydrates under acidic conditions. An amylase produced by fungi and able to hydrolyze carbohydrates under acidic conditions is referred to herein as acid fungal amylase, and is also known as an acid stable fungal  $\alpha$ -amylase. Acid fungal amylase can catalyze the hydrolysis of partially hydrolyzed starch and large oligosaccharides to sugars such as glucose. The acid fungal amylase that can be employed in the present process can be characterized by its ability to aid the hydrolysis of raw or native starch, enhancing the saccharification provided by glucoamylase. In an embodiment, the acid fungal amylase produces more maltose than conventional (e.g., bacterial)  $\alpha$ -amylases.

[0062] Suitable acid fungal amylase can be isolated from any of a variety of fungal species, including *Aspergillus*, *Rhizopus*, *Mucor*, *Candida*, *Coriolus*, *Endothia*, *Entomophthora*, *Irpex*, *Penicillium*, *Sclerotium* and *Torulopsis* species. In an embodiment, the acid fungal amylase is thermally stable and is isolated from *Aspergillus* species, such as *A. niger*, *A. saitoi* or *A. oryzae*, from *Mucor* species such as *M. pusillus* or *M. miehei*, or from *Endothia* species such as *E. parasitica*. In an embodiment, the acid fungal amylase is isolated from *Aspergillus niger*. The acid fungal amylase activity can be supplied as an activity in a glucoamylase preparation, or it can be added as a separate

enzyme. A suitable acid fungal amylase can be obtained from Novozymes, for example in combination with glucoamylase.

[0063] The amount of acid fungal amylase employed in the present process can vary according to the enzymatic activity of the amylase preparation. Suitable amounts include about 0.1 to about 10 acid fungal amylase units (AFAU) per gram of dry solids reduced plant material (e.g., dry solids corn (DSC)). In an embodiment, the reaction mixture can include about 0.3 to about 3 AFAU/gram dry solids reduced plant material (e.g., DSC). In an embodiment, the reaction mixture can include about 1 to about 2 AFAU/gram dry solids reduced plant material (e.g., DSC).

[0064] Glucoamylase

[0065] In certain embodiments, the present method can employ a glucoamylase. Glucoamylase is also known as amyloglucosidase and has the systematic name 1,4- $\alpha$ -D-glucan glucosylhydrolase (E.C. 3.2.1.3). Glucoamylase refers to an enzyme that removes successive glucose units from the non-reducing ends of starch. For example, certain glucoamylases can hydrolyze both the linear and branched glucosidic linkages of starch, amylose, and amylopectin. A variety of suitable glucoamylases are known and commercially available. For example, suppliers such as Novozymes and Genencor provide glucoamylases. The glucoamylase can be of fungal origin.

[0066] The amount of glucoamylase employed in the present process can vary according to the enzymatic activity of the amylase preparation. Suitable amounts include about 0.1 to about 6.0 glucoamylase units (AGU) per gram dry solids reduced plant material (e.g., DSC). In an embodiment, the reaction mixture can include about 1 to about 3 AGU per gram dry solids reduced plant material (e.g., DSC). In an embodiment, the reaction mixture can include about 1 to about 2.5 (e.g., 2.4) AGU per gram dry solids reduced plant material (e.g., DSC). In an embodiment, the reaction mixture can include about 1 to about 2 AGU per gram dry solids reduced plant material (e.g., DSC). In an embodiment, the reaction mixture can include about 1 to about 1.5 AGU per gram dry solids reduced plant material (e.g., DSC). In an embodiment, the reaction mixture can include about 1.2 to about 1.5 AGU per gram dry solids reduced plant material (e.g., DSC).

[0067] Fermenting

[0068] The present process includes fermenting sugars from reduced plant material to ethanol. Fermenting can be effected by a microorganism, such as yeast. The fermentation mixture need not, and in an embodiment does not, include protease. However, the process waters may contain protease. The amount of protease can be less than that used in the conventional process. According to the present invention, fermenting is conducted on a starch composition that has not been cooked. In an embodiment, the present fermentation process produces potable alcohol. Potable alcohol has only acceptable, nontoxic levels of other alcohols, such as fusel oils. Fermenting can include contacting a mixture including sugars from the reduced plant material with yeast under conditions suitable for growth of the yeast and production of ethanol. In an embodiment, fermenting employs the saccharification mixture.

[0069] Any of a variety of yeasts can be employed as the yeast starter in the present process. Suitable yeasts include

any of a variety of commercially available yeasts, such as commercial strains of *Saccharomyces cerevisiae*. Suitable strains include "Fali" (Fleischmann's), Thermosac (Alltech), Ethanol Red (LeSafre), BioFerm AFT (North American Bioproducts), and the like. In an embodiment, the yeast is selected to provide rapid growth and fermentation rates in the presence of high temperature and high ethanol levels. In an embodiment, Fali yeast has been found to provide good performance as measured by final alcohol content of greater than 17% by volume.

[0070] The amount of yeast starter employed is selected to effectively produce a commercially significant quantity of ethanol in a suitable time, e.g., less than 75 hours.

[0071] Yeast can be added to the fermentation by any of a variety of methods known for adding yeast to fermentation processes. For example, yeast starter can be added by as a dry batch, or by conditioning/propagating. In an embodiment, yeast starter is added as a single inoculation. In an embodiment, yeast is added to the fermentation during the fermenter fill at a rate of 5 to 100 pounds of active dry yeast (ADY) per 100,000 gallons of fermentation mash. In an embodiment, the yeast can be acclimated or conditioned by incubating about 5 to 50 pounds of ADY per 10,000 gallon volume of fermenter volume in a prefermenter or propagation tank. Incubation can be from 8 to 16 hours during the propagation stage, which is also aerated to encourage yeast growth. The prefermenter used to inoculate the main fermenter is can be from 1 to 10% by volume capacity of the main fermenter, for example, from 2.5 to 5% by volume capacity relative to the main fermenter.

[0072] In an embodiment, the fermentation is conducted at a pH of about 6 or less, pH of about 3 to about 6, about 3.5 to about 6, about 4 to about 5, about 4 to about 4.5, or about 4.5 to about 5. The initial pH of the fermentation mixture can be adjusted by addition of, for example, ammonia, sulfuric acid, phosphoric acid, process waters (e.g., stillage (back-set), evaporator condensate (distillate), side stripper bottoms, and the like), and the like.

[0073] Although not limiting to the present invention, it is believed that known distillery yeast grow well over the pH range of 3 to 6, but are more tolerant of lower pH's down to 3.0 than most contaminant bacterial strains. Contaminating lactic and acetic acid bacteria grow best at pH of 5.0 and above. Thus, in the pH range of 3.0 to 3.5, it is believed that ethanol fermentation will predominate because yeast will grow better than contaminating bacteria.

[0074] In an embodiment, the present method can include varying the pH. It is believed that varying the pH can be conducted to reduce the likelihood of contamination early in fermentation and/or to increase yeast growth and fermentation during the latter stages of fermentation. For example, fermentation can include filling the fermenter at pH of about 3 to about 4.5 during the first half of fill. Fermentation can include increasing the slurry pH to pH of about 4.5 to about 6 during the second half of the fermenter fill cycle. Fermentation can include maintaining pH by adding fresh substrate slurry at the desired pH as described above. In an embodiment, during fermentation (after filling), pH is not adjusted. Rather, in this embodiment, the pH is determined by the pH of the components during filling.

[0075] In an embodiment, the pH is decreased to about five (5) or below in the corn process waters. In an embodi-

ment, the pH is about pH 4 (e.g. 4.1) at the start of fermentation fill and is increased to about pH 5 (e.g. 5.2) toward the end of fermentation fill. In an embodiment, the method includes stopping pH control of the mash slurry after the yeast culture becomes established during the initial process of filling the fermenter, and then allowing the pH to drift up in the corn process waters during the end stages of filling the fermenter.

[0076] In an embodiment, fermentation is conducted for about to 25 (e.g., 24) to about 150 hours, about 25 (e.g., 24) to about 96 hours, about 40 to about 96 hours, about 45 (e.g., 44) to about 96 hours, about 48 (e.g., 47) to about 96 hours. For example, fermentation can be conducted for about 30, about 40, about 50, about 60, or about 70 hours. For example, fermentation can be conducted for about 35, about 45, about 55, about 65, or about 75 hours.

[0077] In an embodiment, fermentation is conducted at a temperature of about 25 to about 40° C. or about 30 to about 35° C. In an embodiment, during fermentation the temperature is decreased from about 40° C. to about 30° C. or about 25° C., or from about 35° C. to about 30° C., during the first half of the fermentation, and the temperature is held at the lower temperature for the second half of the fermentation. In an embodiment, the temperature can be decreased as ethanol is produced. For example, in an embodiment, during fermentation the temperature can be as high as about 99° F. and then reduced to about 79° F. This temperature reduction can be coordinated with increased ethanol titers (%) in the fermenter.

[0078] In an embodiment, the present method includes solids staging. Solids staging includes filling at a disproportionately higher level of solids during the initial phase of the fermenter fill cycle to increase initial fermentation rates. The solids concentration of the mash entering the fermenter can then be decreased as ethanol titers increase and/or as the fermenter fill cycle nears completion. In an embodiment, the solids concentration can be about 40% (e.g. 41%) during the first half of the fermentation fill. This can be decreased to about 25% after the fermenter is 50% full and continuing until the fermenter fill cycle is concluded. In the above example, such a strategy results in a full fermenter with solids at 33%.

[0079] It is believed that solids staging can accelerate enzyme hydrolysis rates and encourage a rapid onset to fermentation by using higher initial fill solids. It is believed that lowering solids in the last half of fill can reduce osmotic pressure related stress effects on the yeast. By maintaining overall fermenter fill solids within a specified range of fermentability, solids staging improves the capacity of the yeast to ferment high gravity mashes toward the end of fermentation.

#### [0080] Simultaneous Saccharification and Fermentation

[0081] The present process can include simultaneously converting reduced plant material to sugars and fermenting those sugars with a microorganism such as yeast. Simultaneous saccharifying and fermenting can be conducted using the reagents and conditions described above for saccharifying and fermenting.

[0082] In an embodiment, saccharification and fermentation is conducted at a temperature of about 25 to about 40° C. or about 30 to about 35° C. In an embodiment, during

saccharification and fermentation the temperature is decreased from about 40 to about 25° C. or from about 35 to about 30° C. during the first half of the saccharification, and the temperature is held at the lower temperature for the second half of the saccharification.

[0083] Although not limiting to the present invention, it is believed that higher temperatures early during saccharification and fermentation can increase conversion of starch to fermentable sugar when ethanol concentrations are low. This can aid in increasing ethanol yield. At higher ethanol concentrations, this alcohol can adversely affect the yeast. Thus, it is believed that lower temperatures later during saccharification and fermentation are beneficial to decrease stress on the yeast. This can aid in increasing ethanol yield.

[0084] Also not limiting to the present invention, it is believed that higher temperatures early during saccharification and fermentation can reduce viscosity during at least a portion of the fermentation. This can aid in temperature control. It is also believed that lower temperatures later during saccharification and fermentation are beneficial to reduce the formation of glucose after the yeast has stopped fermenting. Glucose formation late in fermentation can be detrimental to the color of the distillers dried grain co-product.

[0085] In an embodiment, saccharification and fermentation is conducted at a pH of about 6 or less, pH of about 3 to about 6, about 3.5 to about 6, about 4 to about 5, about 4 to about 4.5, or about 4.5 to about 5. The initial pH of the saccharification and fermentation mixture can be adjusted by addition of, for example, ammonia, sulfuric acid, phosphoric acid, process waters (e.g., stillage (backset), evaporator condensate (distillate), side stripper bottoms, and the like), and the like.

[0086] In an embodiment, saccharification and fermentation is conducted for about to 25 (e.g., 24) to about 150 hours, about 25 (e.g., 24) to about 72 hours, about 45 to about 55 hours, about 50 (e.g., 48) to about 96 hours, about 50 to about 75 hours, or about 60 to about 70 hours. For example, saccharification and fermentation can be conducted for about 30, about 40, about 50, about 60, or about 70 hours. For example, saccharification and fermentation can be conducted for about 35, about 45, about 55, about 65, or about 75 hours.

[0087] In an embodiment, simultaneous saccharifying and fermenting can be carried out employing quantities of enzyme and yeast selected to maintain high concentrations of yeast and high levels of budding of the yeast in the fermentation broth. For example, the present process can employ quantities of enzyme and yeast selected to maintain yeast at or above about 300 cells/mL or at about 300 to about 600 cells/mL.

[0088] In an embodiment, simultaneous saccharifying and fermenting can be carried out employing quantities of enzyme and yeast selected for effective fermentation without added exogenous nitrogen; without added protease; and/or without added backset. Backset can be added, if desired, to consume process water and reduce the amount of wastewater produced by the process. In addition, the present process maintains low viscosity during saccharifying and fermenting.

[0089] For example, simultaneous saccharifying and fermenting can employ acid fungal amylase at about 0.1 to

about 10 AFAU per gram of dry solids reduced plant material (e.g., DSC) and glucoamylase at about 0.5 to about 6 AGU per gram dry solids reduced plant material (e.g., DSC). For example, simultaneous saccharifying and fermenting can employ acid fungal amylase at about 0.3 to about 3 AFAU per gram of dry solids reduced plant material (e.g., DSC) and glucoamylase at about 1 to about 3 AGU per gram dry solids reduced plant material (e.g., DSC). For example, simultaneous saccharifying and fermenting can employ acid fungal amylase at about 1 to about 2 AFAU per gram of dry solids reduced plant material (e.g., DSC) and glucoamylase at about 1 to about 1.5 AGU per gram dry solids reduced plant material (e.g., DSC).

**[0090]** In an embodiment, simultaneous saccharifying and fermenting can be carried out employing quantities of enzyme and yeast selected to maintain low concentrations of glucose in the fermentation broth. For example, the present process can employ quantities of enzyme and yeast selected to maintain glucose at levels at or below about 2 wt-%, at or below about 1 wt-%, at or below about 0.5 wt-%, or at or below about 0.1 wt-%. For example, the present process can employ quantities of enzyme and yeast selected to maintain glucose at levels at or below about 2 wt-% during saccharifying and fermenting. For example, the present process can employ quantities of enzyme and yeast selected to maintain glucose at levels at or below about 2 wt-% from hours 0-10 (or from 0 to about 15% of the time) of saccharifying and fermenting. For example, the present process can employ quantities of enzyme and yeast selected to maintain glucose at levels at or below about 1 wt-%, at or below about 0.5 wt-%, or at or below about 0.1 wt-% from hours 12-54 (or from about 15% to about 80% of the time) of saccharifying and fermenting. For example, the present process can employ quantities of enzyme and yeast selected to maintain glucose at levels at or below about 1 wt-% from hours 54-66 (or about from 80% to about 100% of the time) of saccharifying and fermenting. Suitable levels of enzyme include acid fungal amylase at about 0.3 to about 3 AFAU per gram of dry solids reduced plant material (e.g., DSC) and glucoamylase at about 1 to about 3 AGU per gram dry solids reduced plant material (e.g., DSC). For example, simultaneous saccharifying and fermenting can employ acid fungal amylase at about 1 to about 2 AFAU per gram of dry solids reduced plant material (e.g., DSC) and glucoamylase at about 1 to about 1.5 AGU per gram dry solids reduced plant material (e.g., DSC).

**[0091]** In an embodiment, simultaneous saccharifying and fermenting can be carried out employing quantities of enzyme and yeast selected to maintain low concentrations of maltose (DP2) in the fermentation broth. For example, the present process can employ quantities of enzyme and yeast selected to maintain maltose at levels at or below about 0.5 wt-% or at or below about 0.2 wt-%. Suitable levels of enzyme include acid fungal amylase at about 0.3 to about 3 AFAU per gram of dry solids reduced plant material (e.g., DSC) and glucoamylase at about 1 to about 3 AGU per gram dry solids reduced plant material (e.g., DSC). For example, simultaneous saccharifying and fermenting can employ acid fungal amylase at about 1 to about 2 AFAU per gram of dry solids reduced plant material (e.g., DSC) and glucoamylase at about 1 to about 1.5 AGU per gram dry solids reduced plant material (e.g., DSC).

**[0092]** In an embodiment, simultaneous saccharifying and fermenting can be carried out employing quantities of enzyme and yeast selected to maintain low concentrations of dextrin in the fermentation broth. For example, the present process can employ quantities of enzyme and yeast selected to maintain maltotriose (DP3) at levels at or below about 0.5 wt-%, at or below about 0.2 wt-%, or at or below about 0.1 wt-%. For example, the present process can employ quantities of enzyme and yeast selected to maintain dextrin with a degree of polymerization of 4 or more (DP4+) at levels at or below about 1 wt-% or at or below about 0.5 wt-%. Suitable levels of enzyme include acid fungal amylase at about 0.3 to about 3 AFAU per gram of dry solids reduced plant material (e.g., DSC) and glucoamylase at about 1 to about 3 AGU per gram dry solids reduced plant material (e.g., DSC). For example, simultaneous saccharifying and fermenting can employ acid fungal amylase at about 1 to about 2 AFAU per gram of dry solids reduced plant material (e.g., DSC) and glucoamylase at about 1 to about 1.5 AGU per gram dry solids reduced plant material (e.g., DSC).

**[0093]** In an embodiment, simultaneous saccharifying and fermenting can be carried out employing quantities of enzyme and yeast selected to maintain low concentrations of fusel oils in the fermentation broth. For example, the present process can employ quantities of enzyme and yeast selected to maintain fusel oils at levels at or below about 0.4 to about 0.5 wt-%. Suitable levels of enzyme include acid fungal amylase at about 0.3 to about 3 AFAU per gram of dry solids reduced plant material (e.g., DSC) and glucoamylase at about 1 to about 3 AGU per gram dry solids reduced plant material (e.g., DSC). For example, simultaneous saccharifying and fermenting can employ acid fungal amylase at about 1 to about 2 AFAU per gram of dry solids reduced plant material (e.g., DSC) and glucoamylase at about 1 to about 1.5 AGU per gram dry solids reduced plant material (e.g., DSC).

**[0094]** Additional Ingredients for Saccharification and/or Fermentation

**[0095]** The saccharification and/or fermentation mixture can include additional ingredients to increase the effectiveness of the process. For example, the mixture can include added nutrients (e.g., yeast micronutrients), antibiotics, salts, added enzymes, and the like. Nutrients can be derived from stillage or backset added to the liquid. Suitable salts can include zinc or magnesium salts, such as zinc sulfate, magnesium sulfate, and the like. Suitable added enzymes include those added to conventional processes, such as protease, phytase, cellulase, hemicellulase, exo- and endo-glucanase, xylanase, and the like.

**[0096]** Recovering Ethanol from the Beer

**[0097]** The product of the fermentation process is referred to herein as "beer". For example, fermenting corn produces "corn beer". Ethanol can be recovered from the fermentation mixture, from the beer, by any of a variety of known processes. For example, ethanol can be recovered by distillation.

**[0098]** The remaining stillage includes both liquid and solid material. The liquid and solid can be separated by, for example, centrifugation. The recovered liquid, thin stillage, can be employed as at least part of the liquid for forming the saccharification and fermentation mixture for subsequent batches or runs.

[0099] The recovered solids, distiller's dried grain, include unfermented grain solids and spent yeast solids. Thin stillage can be concentrated to a syrup, which can be added to the distiller's dried grain and the mixture then dried to form distiller's dried grain plus solubles. Distiller's dried grain and/or distiller's dried grain plus solubles can be sold as animal feed.

**[0100] Burn-Out of Residual Starches for Subsequent Fermentation**

[0101] In an embodiment, the present method can include heat treatment of the beer or stillage, e.g., between the beer well and distillation. This heat treatment can convert starches to dextrins and sugars for subsequent fermentation in a process known as burn-out. Such a treatment step can also reduce fouling of distillation trays and evaporator heat exchange surfaces. In an embodiment, heat treatment staging can be performed on whole stillage. Following enzymatic treatment of the residual starches, in an embodiment, the resulting dextrins and sugars can be fermented within the main fermentation process as recycled backset or processed in a separate fermentation train to produce ethanol.

**[0102] Fractionation of Solids from Fermentation**

[0103] Large pieces of germ and fiber can ferment the residual starch in the fermenter. After fermentation, the fractions could be removed prior to or after distillation. Removal can be effected with a surface skimmer before to distillation. In an embodiment, screening can be performed on the beer. The screened material can then be separated from the ethanol/water mix by, for example, centrifugation and rotary steam drum drying, which can remove the residual ethanol from the cake. In embodiments in which the larger fiber and germ pieces are removed prior to bulk beer distillation, a separate stripper column for the fiber/germ stream can be utilized. Alternatively, fiber and germ could be removed by screening the whole stillage after distillation.

[0104] In an embodiment, all the components are blended and dried together. The fiber and germ can be removed from the finished product by aspiration and/or size classification. The fiber from the DDGS can be aspirated. Removal of fiber by aspiration after drying increased the amount of oil and protein in the residual DDGS by 0.2 to 1.9% and 0.4 to 1.4%, respectively. The amount of NDF in the residual DDGS decreased by 0.1 to 2.8%.

[0105] In an embodiment, fractionation can employ the larger fiber and germ pieces to increase the particle size of that part of the DDGS derived from the endosperm, as well as to improve syrup carrying capacity. A ring dryer disintegrator can provide some particle size reduction and homogenization.

**[0106] Continuous Fermentation**

[0107] The present process can be run via a batch or continuous process. A continuous process includes moving (pumping) the saccharifying and/or fermenting mixtures through a series of vessels (e.g., tanks) to provide a sufficient duration for the process. For example, a multiple stage fermentation system can be employed for a continuous process with 48-96 hours residence time. For example, reduced plant material can be fed into the top of a first vessel for saccharifying and fermenting. Partially incubated and

fermented mixture can then be drawn out of the bottom of the first vessel and fed in to the top of a second vessel, and so on.

[0108] Although not limiting to the present invention, it is believed that the present method is more suitable than conventional methods for running as a continuous process. It is believed that the present process provides reduced opportunity for growth of contaminating organisms in a continuous process. At present, the majority of dry grind ethanol facilities employ batch fermentation technology. This is in part due to the difficulty of preventing losses due to contamination in these conventional processes. For efficient continuous fermentation using traditional liquefaction technology, the conventional belief is that a separate saccharification stage prior to fermentation is necessary to pre-saccharify the mash for fermentation. Such pre-saccharification insures that there is adequate fermentable glucose for the continuous fermentation process.

[0109] The present method achieves efficient production of high concentrations of ethanol without a liquefaction or saccharification stage prior to fermentation. This is surprising since this conventional wisdom teaches that it is necessary to have adequate levels of fermentable sugar available during the fermentation process when practiced in a continuous mode. In contrast the present method can provide low concentrations of glucose and efficient fermentation. In the present method, it appears that the glucose is consumed rapidly by the fermenting yeast cell. It is believed that such low glucose levels reduce stress on the yeast, such as stress caused by osmotic inhibition and bacterial contamination pressures. According to the present invention, ethanol levels greater than 18% by volume can be achieved in about to about 96 hours.

**[0110] High Alcohol Beer**

[0111] The present invention also relates to a high alcohol beer. In an embodiment, the process of the present invention produces beer containing greater than 18 vol-% ethanol. The present process can produce such a high alcohol beer in about 40 to about 96 hours or about to about 96 hours. In an embodiment, the beer includes 18 vol-% to about 23 vol-% ethanol. For example, the present method can produce alcohol contents in the fermenter of 18 to 23% by volume in about 45 to 96 hours.

[0112] By way of further example, the present method can produce alcohol content in the fermenter of 18 to 23% by volume in about 45 to 96 hours. In certain embodiments, the majority of the alcohol (80% or more of the final concentration) is produced in the first 45 hours. Then, an additional 2 to 5 vol-% alcohol can be produced in the final 12-48 hours. Concentrations of ethanol up to 23 vol-% can be achieved with fermentation time up to 96 hours. It can be economically advantageous to harvest after 48 to 72 hours of fermentation to increase fermenter productivity.

[0113] The present beer can include this high level of ethanol even when it includes high levels of residual starch. For example, the present beer can include ethanol at 18 to 23 vol-% when it contains 0 to 30% residual starch. The present beer can contain residual starches as low as 0% to as high as 20% residual starch.

[0114] By conventional measures, high levels of residual starch indicate inefficient fermentation, which yields only

low levels of ethanol. In contrast, although not limiting to the present invention, it is believed that the present method results in fewer Maillard type reaction products and more efficient yeast fermentation (e.g., reduced levels of secondary metabolites). This is believed to be due to the low glucose levels and low temperatures of the present method compared to conventional saccharification and liquefaction. Thus, the present method can produce more alcohol even with higher levels of residual starch.

[0115] In an embodiment, the present beer includes fewer residual byproducts than conventional beers, even though residual starch can be higher. For example, residual glucose, maltose, and higher dextrins (DP3+) can be as much as 0.8 wt-% lower than in conventional beers produced under similar fermentation conditions. By way of further example, residual glycerol can be as much as 0.45 wt-% less. Lactic acid and fusel oils can also be significantly reduced. For example, the present beer can include less than or equal to about 0.2 wt-% glucose, about 0.4 wt-%, about 0.1 wt-% DP3, undetectable DP4+, 0.45 wt-% glycerol, about 0.01 wt-% lactic acid, and/or about 0.4 wt-% fusel oils.

[0116] Distiller's Dried Grain

[0117] High Protein Distiller's Dried Grain

[0118] The present invention also relates to a distiller's dried grain product. The distiller's dried grain can also include elevated levels of one or more of protein, fat, fiber (e.g., neutral detergent fiber (NDF)), and starch. For example, the distiller's dried grain can include 34 or more wt-% protein or about 30 to about 45 wt-% protein, or about 1 to about 2 wt-% more protein than produced by the conventional process. For example, the distiller's dried grain can include 15 or more wt-% fat, about 13 to about 17 wt-% fat, or about 1 to about 6 wt-% more fat than produced by the conventional process. For example, the distiller's dried grain can include 31 or more wt-% fiber, about 23 to about 37 wt-% fiber, or about 3 to about 13 wt-% more fiber than produced by the conventional process. For example, the distiller's dried grain can include 12 or more wt-% starch, about 1 to about 23 wt-% starch, or about 1 to about 18 wt-% more starch than produced by the conventional process.

[0119] In an embodiment, the present distiller's dried grain includes elevated levels of B vitamins, vitamin C, vitamin E, folic acid, and/or vitamin A, compared to conventional distiller's dried grain products. The present distiller's dried grain has a richer gold color compared to conventional distiller's dried grain products.

[0120] Distiller's Dried Grain With Improved Physical Characteristics

[0121] The present invention also relates to a distiller's dried grain with one or more improved physical characteristics, such as decreased caking or compaction or increase ability to flow. The present process can produce such an improved distiller's dried grain.

[0122] Although not limiting to the present invention, it is believed that the present process can produce fermentation solids including higher molecular weight forms of carbohydrates. Such fermentation solids can, it is believed, exhibit a higher glass transition temperature (i.e. higher  $T_g$  values). For example, residual starches have a high  $T_g$  value. Thus,

through control of starch content in the DDG and DDGS, the present process can manufacture DDG or DDGS with target  $T_g$  values.

[0123] Further, according to the present invention, adding an alkaline syrup blend (e.g., syrup plus added lime or other alkaline material) to the fermentation solids (e.g., distiller's dried grains) can provide decreased caking or compaction or increase ability to flow to the distiller's dried grain with solubles (DDGS).

[0124] Although not limiting to the present invention, it is believed that organic acids such as lactic, acetic, and succinic acids which are produced in fermentation have a lower  $T_g$  value than their corresponding calcium salts. Maintenance of residual carbohydrate in higher molecular weight form, or addition of lime to form calcium salts of organic acids, are two strategies for forming higher  $T_g$  value co-products that will be less likely to undergo the glass transition, resulting in the deleterious phenomenon known as caking.

[0125] Although not limiting to the present invention, it is believed that process of the present invention can need not destroy protein in the fermented plant material. Corn contains prolamins, such as zein. Grain sorghum, for example, contains a class of zein-like proteins known as kafirins, which resemble zein in amino acid composition. The thermal degradation that occurs during liquefaction, distillation, and high temperature drying produces DDG and DDGS including significant amounts of degraded protein. It is believed that the process of the present invention can provides improved levels of the prolamin fraction of cereal grains.

[0126] It is believed that extended exposure to high alcohol concentrations that can be achieved by the present process can condition the proteins in the plant material. This can solubilize some of the proteins. For example, it is believed that in distillation the ethanol concentration reaches levels that can solubilize prolamins (e.g., zein) in the beer. Upon the removal, or "stripping," of ethanol from the beer, prolamins (such as zein) can be recovered concentrated in DDG and DDGS. The resulting high protein content of DDG and DDGS can be advantageous for various end uses of DDG and DDGS, for example in further processing or compounding.

[0127] In an embodiment, efficient fermentation of the present process removes from the DDG or DDGS non zein components such as starch. Fractionating the plant material, e.g., corn, can also increase levels of proteins, such as zein, in the DDG or DDGS. For example, removing the bran and germ fractions prior to fermentation can concentrate zein in the substrate. Zein in corn is isolated in the endosperm. Fermentation of zein enriched endosperm results in concentration of the zein in the residuals from fermentation.

[0128] In an embodiment, the process of the present invention can provide DDG and DDGS with different, predetermined  $T_g$  values. The process of the present invention can ferment fractions containing high, medium, or low levels of zein, thus varying the glass transition temperature of the resulting DDG or DDGS. The resulting co-product  $T_g$  can be directly proportional to the prolamin protein (such as zein) content. The process of the current invention is desirable for the fermentation of high protein corn. This also allows production of DDG and DDGS with a higher prolamin (zein) content.

[0129] Residual starch remaining at the end of fermentation preferentially segregates into the thin stillage fraction, which is subsequently evaporated to produce syrup. The wet cake fraction produced by the present method, which can be dried separately to produce DDG, can be higher in prolamin protein (such as zein) than conventional DDG. The present process allows syrup and wet cake blend ratios to be varied. This results in DDG/DDGS with varying ratios of prolamin protein (such as zein) and residual starch. As the residual starch in the wet cake reduces the protein in the wet cake increases. This indicates an inverse relationship. A similar response occurs in the syrup fraction.

[0130] It is believed that starch can segregate into the liquid fraction. The amount of starch in the DDGS can be varied by blending syrup at rates ranging from 0 lbs. dry weight of syrup solids to 1.2 lbs. of syrup solids per lb. of wet cake solids before, and various times during drying to create the final DDGS product. The disproportionate segregation of residual starches into the backset or thin stillage fraction can provide both the aforementioned burn-out and secondary fermentation to be performed on these fractions. Since the thin stillage is evaporated to produce syrup, the centrifuge mass balance also enables DDGS production at various  $T_g$  values depending on the desired properties and their dependence on  $T_g$ .

#### [0131] Emissions

[0132] The present invention has emissions benefits. Emissions benefits result in the reduction in byproducts created in the ethanol manufacturing process. There is a marked reduction in extraction of fats and oils in the mash from the germ fraction of cereal grains. There is a reduction of byproducts from Maillard reactions typically formed during cooking and liquefaction. And there is a reduction in fermentation byproducts. These observations result in reduced emissions during the recovery of co-products. The concentration and emission rates of volatile organic compounds (VOC), carbon monoxide (CO), nitric oxide compounds (NO<sub>x</sub>), sulfur oxides (SO<sub>2</sub>), and other emissions are considerably lower. See Table 1. Note that other manufacturers have attempted to lower emissions by manufacturing wet cake instead of drying to DDG or DDGS.

[0133] The present invention also relates to volatile organic compounds (VOC), such as those produced by drying products of a fermentation process. The present method includes producing ethanol, distiller's dried grain, and additional useful fermentation products with production of lower levels of VOC compared to conventional processes. For example, in the present method, drying distillation products (e.g., spent grain) produces reduced levels of VOC.

[0134] Conventional fermentation processes using corn, for example, produces about 2.1 pounds of VOC's from drying distillation products from each ton of corn processed. The actual stack emissions can be less due to pollution control equipment. The present method results in at least 30% reduction in VOC production to about 1.47 or less pounds per ton of corn processed. These emissions reductions are unexpected yet highly significant, and provide for more efficient use of emissions reduction control technology, such as thermal oxidizers.

[0135] VOC produced by fermentation processes include ethanol, acetic acid, formaldehyde, methanol, acetaldehyde, acrolein, furfural, lactic acid, formic acid, and glycerol.

[0136] The present invention also relates to carbon monoxide (CO), such as those produced by drying products of a fermentation process. The present method includes producing ethanol, distiller's dried grain, and additional useful fermentation products with production of lower levels of CO compared to conventional processes. For example, in the present method, drying distillation products (e.g., spent grain) produces reduced levels of CO.

[0137] Conventional fermentation processes using corn, for example, produces about 1.4 pounds of CO's from drying distillation products from each ton of corn processed. The actual stack emissions can be less due to pollution control equipment. The present method results in a 30% reduction in CO production to about 0.98 or less pounds per ton of corn processed. These emissions reductions are unexpected yet highly significant, and provide for more efficient use of emissions reduction control technology, such as thermal oxidizers.

TABLE 1

		<u>Emissions Reductions</u>			
Emission Type	Units	Conventional Run	Inventive Process	Emissions Reduction %	
VOC	Concentration	ppmv	663	459.65	30.67
	Emission Rate	lb/dscf	13.35	7.91	40.75
CO	Concentration	ppmv	434	234.13	46.05
	Emission Rate	lb/dscf	9.1	4.94	45.71

[0138] The present invention may be better understood with reference to the following examples. These examples are intended to be representative of specific embodiments of the invention, and are not intended as limiting the scope of the invention.

## EXAMPLES

### Example 1

#### Production of Improved Distiller's Dried Grain from Corn

[0139] A method according to the present invention was employed to produce distiller's dried grain from corn. This method produced high protein, high fat, and high fiber distiller's dried grain. Comparison with a conventional saccharification and liquefaction process indicates superior performance of the present method.

#### [0140] Materials and Methods

#### [0141] Raw Starch Fermentation

[0142] Yeast inoculum was prepared by adding glucoamylase (0.088 ml of Novozyme's Spirizyme Plus gluco-amylase at 400 AGU/g) and protease (0.018 ml of Genencor International's GC 106 protease 1000 SAPU/g) to 400 ml of stillage containing 70 grams of maltodextrin. Stillage (backset) used was prepared from prior conventional or raw starch fermentations by distilling off the alcohol and subjecting the resulting whole stillage to centrifugal separation to produce

backset. 1.07 grams of urea, 0.13 grams of zinc sulfate, and 0.00067 ml of a 1:1000 dilution of Antibiotic (Alltech Lactocide. [amount?])mg were also added. About 300-400 million cells/ml of viable cells of yeast (*Saccharomyces cerevisiae*) (0.48 g of Fleischmann's Fali yeast) was added to this mixture and propagation was conducted without stirring, or agitating, for 8 hours at an incubation temperature of 90° F. Flasks were periodically swirled under gentle conditions to effect mixing of the contents. The resulting yeast culture (10.8 ml) was added directly to each fermenter for inoculation.

[0143] Corn was obtained from commercial suppliers of seed corn and was ground through a 0.5 mm screen using a hammermill prior to fermentation. Several varieties of conventional number 2 yellow dent corn were compared, and in several experiments their isogenically equivalent of waxy corn was also tested. Different corn varieties were tested to demonstrate that the present methods produce improved DDG using any of a variety of corn hybrids.

[0144] Approximately 129 to 134 grams of the appropriate corn was mixed in about 225 ml of water. Actual grams of flour (ground corn) and water volumes were adjusted for each fermenter based on the moisture content of the flour so that all fermentations were run at approximately 33.4 grams of dry solids corn per 100 grams of water (33.4% DSC). All raw starch fermenters were adjusted to pH 5.0 with sulfuric acid.

[0145] Fermentations were conducted at 82° F. Antibiotic (Alltech Lactocide. 3 mg) was added to each fermentation batch. The raw starch fermentations employed a commercially available glucoamylase preparation (Novozymes' Spizzyme Plus 0.317 ml of GAU/ml) which also includes acid fungal amylase activity.

[0146] Fermentations were conducted for 72 hours with sampling conducted at approximately 24 (e.g. 25) hour intervals. All samples were analyzed by HPLC. At the end of fermentation beer samples were placed in metal pans, pH was decreased to <3.5 to inactivate residual enzyme activity, and dried.

[0147] Conventional Fermentation

[0148] Preparation of yeast inoculum and grinding of corn to corn flour was accomplished as described above for the raw starch fermentation.

[0149] For fermentations employing the conventional process, pH adjustment was not necessary; the natural pH of the

water and corn flour was 5.8 to 6.0. The conventional fermentations started with a saccharification or cooking stage to liquefy the starch in the mixture. The cook stage was conducted for 60 minutes at a temperature of 85° C. 0.044 ml of Novozymes Liquezyme SC Alpha-amylase (0.044 ml of Novozymes Liquezyme SC 120 AFAU (KNU)/ml) was added to liquefy the corn mash.

[0150] Conventional fermentations were also run at 82° F. and included Antibiotic (3 mg of Alltech Lactocide antibiotic). Protease (0.0047 ml of GC 106 protease (1000 SAPU/g/ml) and 0.64 ml of 50% urea liquor (50% of industrial grade urea) were added to fermenters using the conventional process. A commercially available glucoamylase (0.095 ml of Genencor International's GC 480 glucoamylase at 400 AGU/ml) was added for fermentation. Otherwise, fermentations were generally conducted as described above for raw starch fermentations.

[0151] Results and Discussion

[0152] Fermentation Results are shown in Table 1 and summarized in Table 2.

TABLE 1A

Comparison of Process Impacts on Proximate Analysis of DDGS				
Corn Hybrid	Residual Sugars as Glucose (%)		% Acids Lactic & Acetic	
	Conv	RSH	Conv	RSH
#2 Yellow Hybrid A	2.57	0.58	0.09	0.06
#2 Yellow Hybrid B	1.67	0.84	0.09	0.06
Waxy Isogenic Pair to Hybrid B	1.70	2.11	0.10	0.06
#2 Yellow Hybrid C	1.18	0.62	0.08	0.06
Waxy Isogenic Pair to Hybrid C	1.43	1.49	0.10	0.07
#2 Yellow Hybrid D	0.84	0.49	0.06	0.05
Waxy Isogenic Pair to Hybrid D	0.58	0.89	0.06	0.07
Waxy Hybrid E	1.15	0.50	0.10	0.06
#2 Yellow Hybrid F	1.86	0.61	0.11	0.07
Waxy Hybrid G	1.23	0.97	0.12	0.09
Hetero Waxy Isogenic Pair to Hybrid G	1.14	0.39	0.10	0.07
Averages	1.40	0.86	0.09	0.07

[0153]

TABLE 1B

Comparison of Process Impacts on Proximate Analysis of DDGS										
Corn Hybrid	% Glycerol		% Starch		% Protein		% Fat		% NDF	
	Conv	RSH	Conv	RSH	Conv	RSH	Conv	RSH	Conv	RSH
#2 Yellow Hybrid A	1.09	0.86	6.86	22.24	31.25	32.15	11.05	13.65	20.45	29.00
#2 Yellow Hybrid B	1.12	0.77	2.78	21.14	31.90	33.20	13.30	17.00	24.90	32.30
Waxy Isogenic Pair to Hybrid B	1.11	0.75	1.97	14.35	31.10	30.40	14.30	16.40	25.30	34.10
#2 Yellow Hybrid C	1.20	0.85	1.68	17.51	31.50	33.80	15.00	21.30	22.00	31.00

TABLE 1B-continued

	Comparison of Process Impacts on Proximate Analysis of DDGS									
	% Glycerol		% Starch		% Protein		% Fat		% NDF	
	Conv	RSH	Conv	RSH	Conv	RSH	Conv	RSH	Conv	RSH
Corn Hybrid										
Waxy Isogenic Pair to Hybrid C	1.13	0.82	1.79	9.92	30.00	29.70	15.20	17.10	24.60	37.40
#2 Yellow Hybrid D	1.03	0.74	0.83	14.61	36.40	37.60	11.90	14.80	23.40	28.90
Waxy Isogenic Pair to Hybrid D	1.06	0.78	1.11	3.39	33.30	34.20	12.80	15.70	24.60	31.70
Waxy Hybrid E	1.11	0.76	0.65	1.90	35.60	35.90	11.60	13.30	26.90	29.90
#2 Yellow Hybrid F	1.17	0.78	3.27	15.99	31.80	31.10	12.50	13.30	28.10	33.10
Waxy Hybrid G	1.11	0.84	10.49	1.04	39.70	41.10	12.10	14.00	20.30	23.70
Hetero Waxy Isogenic Pair to Hybrid G	1.05	0.84	12.15	13.74	36.60	38.90	8.96	10.90	20.80	26.50
Averages	1.11	0.80	3.96	12.35	33.56	34.37	12.61	15.22	23.76	30.69

[0154]

TABLE 2

Proximate Analysis	Comparison of Process Impacts on Proximate Analysis of DDGS (Summary)	
	Process	
	Conventional	Raw Starch
Starch	3.96	12.35
Protein	33.56	34.37
Fat	12.61	15.22
Fiber	23.76	30.69
Ash	4.06	4.29
Unknown	22.05	3.08
Summation	100.00	100.00

[0155] An interesting feature of the raw starch process is that it results in distiller's dried grain with solubles (DDGS) with equal or higher levels of several components, even when it appears that fermentation efficiency, as measured by residual starch, was decreased for the raw starch process. One would expect that, with the lower efficiency, the other components of the DDGS would be lower based on mass balance. The raw starch process apparently results in less damage to the constituents of the grain.

[0156] Another interesting feature of the raw starch process is the performance improvement realized using waxy corn hybrids. Waxy corn is almost entirely comprised of amylopectin starch, whereas normal #2 yellow corn is about 25 to 28% amylose starch with the remainder being amylopectin. Waxy corn is generally not used in the conventional process because of the high peak viscosity and more rapid rate of viscosity development compared to regular corn. The high initial viscosity makes the corn slurry more difficult to pump during the initial primary high temperature liquefaction. Waxy corn varieties can, however, be readily employed in the present process. Because no cook stage is employed, the high peak viscosity is not a processing issue.

Example 2

#### The Present Process Provides Improved Yield Potential

[0157] The yield potential of the method of the present invention was compared to a conventional process. The present method exhibited improved yield using temperature staging. The present method exhibited an increased potential maximum yield for ethanol production. Comparison with conventional saccharification and liquefaction process indicates superior performance of the present method.

#### [0158] Materials and Methods

[0159] Fermentations were prepared in a similar manner as in Example 1 except for intentional differences in particle size, alpha amylase enzyme dose, gluco-amylase enzyme dose, or acid fungal amylase enzyme dose. Conditions for this experiment are described in Table 3. Corn for all tests was obtained from Broin Enterprises (BEI), Scotland, S. Dak., USA. Corn representing a coarse particle size by raw starch standards was ground at BEI. Finely ground corn was produced using a lab hammermill through a 0.5 mm screen.

[0160] The conventional process utilized indicated levels of Liquezyme SC and GC 480. The raw starch process used indicated levels of Spirizyme Plus and SP 288 acid fungal amylase at 1700 AFAU's per gm. Dosages of urea liquor, zinc sulfate, and antibiotic were adjusted accordingly for the conventional process. Stillage (backset) used was prepared from prior conventional or raw starch fermentations by distilling off the alcohol and subjecting the resulting whole stillage to centrifugal separation to produce backset. Fermentation temperatures were staged according to the following set points: 0-18 hours at 90° F., 18-42 hours at 86° F., and 42-72 hours at 82° F. Samples were taken at 65 hours to represent the end of fermentation.

#### [0161] Results and Discussion

[0162] The objective of these experiments was to illustrate the sensitivities of the two processes to changes in enzyme dose rate and compare differences in ethanol % and residual starch. The results are shown in Table 3 and FIGS. 1A, 1B, 1C, 1D and 1E. The impact of grind size and enzyme dose on the two processes is apparent. Note that SP 288 acid

fungus amylase is effective at accessing raw starch. Acid fungus amylase appears to improve the ability to access starch such that grind size has less effect on yield when SP 288 is present. The present process achieved significantly better alcohol yields at equivalent or higher residual starch levels. FIG. 1B illustrates a similar effect of grind size on ethanol yield in the conventional process, and demonstrates the importance of GA dosage level on accessing starch in coarse grain particles.

[0163] Extrapolation of the results for both the conventional and raw starch process shown in FIGS. 1A and 1B to zero residual starch reveals an embodiment of the raw starch process. As residual starch levels decrease based on improving conversion efficiencies, this process can achieve higher ethanol % than the conventional process. For example, in the absence of residual starch, the present process in this example would produce 21.3 vol-% ethanol, but the conventional process would produce only 20.6 vol-% ethanol. Such an increase is significant. The present process potential of the new process compared to the existing process is shown in FIGS. 1C and 1D. These figures summarize the results for both processes run under the varying grind size and enzyme dosage combinations. FIG. 1C illustrates the potential for the new process to produce more alcohol than

the conventional process, even when residual starch levels are higher. Conventional wisdom would suggest the raw starch process is less efficient due to the higher levels of residual starch, however, this is not the case. The present process is superior to the conventional method. Note that fermentation efficiency can also be assessed by examining the fermentation drop solids. This is shown in the composite data comparing both processes in FIG. 1D. Since all fermentations in the above example were started at the same initial set solids, a lower drop solids suggests a more efficient conversion of starch to ethanol. The potential of this process is also indicated by the achievement of an equal to or reduced level of drop solids, despite the higher residual starches observed.

[0164] FIG. 1E shows the temperature staging done during the present process. Fermentation temperatures were staged according to the following set points: 0-18 hours at approximately 90° F. (ranging from about 95° F. to about 90° F.), 18-42 hours at approximately 86° F. (ranging from 90° F. to 86° F.), and 42-72 hours at about 82° F. (ranging from 86° F. to 84° F.). Staging of temperature helps to increase ethanol production process by reducing stress on yeast. The temperature is decreased as ethanol is produced to reduce the stress on yeast caused by ethanol production.

TABLE 3

Comparison of the Yield Potential of Conventional vs. Raw Starch Processes									
Conventional Fermentation Process									
Grind Used	Enzyme Dosages		Process Water Amounts		Corn		Slurry		Residual Starch Dry Wt. %
	AA (ml)	GA (ml)	Water (ml)	Backset %	Flour Wt. %	Dry Solids	AA Dose	Ethanol Vol %	
BEI	0.04	0.08	285	40	190	35.91	Low	16.21	19.49
BEI	0.04	0.12	285	40	190	35.89	Low	17.57	14.69
BEI	0.06	0.08	285	40	190	35.90	Medium	16.22	15.14
BEI	0.06	0.12	285	40	190	35.89	Medium	17.12	14.03
BEI	0.08	0.08	285	40	190	35.89	High	15.93	16.72
BEI	0.08	0.12	285	40	190	35.88	High	17.47	12.78
0.5 mm	0.04	0.08	295	40	176	35.85	Low	16.78	15.64
0.5 mm	0.04	0.12	295	40	176	35.83	Low	18.40	9.58
0.5 mm	0.06	0.08	295	40	176	35.84	Medium	16.57	15.77
0.5 mm	0.06	0.12	295	40	176	35.83	Medium	18.19	10.36
0.5 mm	0.08	0.08	295	40	176	35.83	High	16.92	16.48
0.5 mm	0.08	0.12	295	40	176	35.82	High	18.31	9.27
Raw Starch Fermentation Process									
Grind Used	Enzyme Dosages		Process Water Amounts		Corn		Slurry		Residual Starch Dry Wt. %
	AA (ml)	GA (ml)	Water (ml)	Backset %	Flour Wt. %	Dry Solids	GA Dose	Ethanol Vol %	
BEI	0.00	0.34	285	40	190	36.35	Low	17.53	22.37
BEI	0.03	0.34	285	40	190	36.35	Low	19.19	14.45
BEI	0.00	0.42	285	40	190	36.32	Medium	17.82	19.65
BEI	0.03	0.42	285	40	190	36.32	Medium	19.14	11.15
BEI	0.00	0.53	285	40	190	36.28	High	18.11	19.83
BEI	0.03	0.53	285	40	190	36.28	High	19.13	12.80
0.5 mm	0.00	0.34	295	40	176	36.31	Low	18.20	19.30
0.5 mm	0.03	0.34	295	40	176	36.31	Low	19.22	13.54
0.5 mm	0.00	0.42	295	40	176	36.28	Medium	18.51	17.24
0.5 mm	0.03	0.42	295	40	176	36.28	Medium	19.56	10.50
0.5 mm	0.00	0.53	295	40	176	36.24	High	18.75	16.38

TABLE 3-continued

Comparison of the Yield Potential of Conventional vs. Raw Starch Processes								
Screen Size (mm)	No. 12	No. 16	No. 20	No. 25	No. 30	No. 35	Pan	Sieve Size
BEI Grind	1.70 mm	1.18 mm	0.85 mm	0.71 mm	0.60 mm	0.50 mm	<0.50 mm	Pore Size (mm)
0.5 mm	0.02	0.26	2.53	7.91	12.14	20.80	54.96	Percentage on Pan
	0.00	0.00	0.00	0.00	0.00	0.00	100.00	
Process		AA			GA			
Conventional Raw Starch		Liquozyme SC SP 288			GC 480 Spirizyme Plus			

## Example 3

The Present Process Exhibits Improved Results with Increased Levels of Acid Fungal Amylase and Increased Levels of Glucoamylase

[0165] Results of an embodiment of the method of the present invention were evaluated with increased levels of acid fungal amylase and increased levels of glucoamylase. Increased levels of acid fungal amylase improved results with the present process. The increased levels of glucoamylase improved results with the present process.

## [0166] Materials and Methods

[0167] Glucoamylase (Novozymes Spirizyme Plus) and acid fungal amylase (Novozymes SP 288) were both tested in raw starch fermentations in a manner similar to Example 2, using the coarser grind.

## [0168] Results and Discussion

[0169] The objective of this test was to examine the effect of a range of dosages of glucoamylase and acid fungal amylase on production of ethanol and other products from raw starch hydrolysis fermentations. In particular, dosages above 0.3 AFAU's per gm dry solids corn for acid fungal amylase and dosages above 0.3 AGU's per gram of dry solids corn produce higher alcohol and consistently higher

residual glucose. The consistently higher glucose indicates that these fermentations have the potential for even higher ethanol yield.

[0170] These results suggest that glucoamylase and acid fungal amylase acted synergistically to access raw starch and convert the starch to fermentable sugar. See FIGS. 2A, 2B, and 2C.

## Example 4

Impact of Grinding or Reducing Grain Particle Size on Fermentation Efficiency

[0171] Results of an embodiment of the method of the present invention were evaluated with varying particle size of the ground plant material. Smaller particle sizes improved results with the present process.

## [0172] Materials and Methods

[0173] A series of lab scale hammermill grinds were performed to generate flour ranging from coarse to relatively fine particle sizes. Raw starch fermentations were set up in a similar manner as in Example 2. Corn Flour used as substrate was ground through a lab hammermill to pass through screens of 0.5 mm, 2.0 mm, and 2.4 mm openings. The conditions tested are shown in Table 4.

TABLE 4

Impact of Grind Particle Size and Glucoamylase Dosage on Fermentation Efficiency												
Sieve Results							Particle					
Grind	No. 12	No. 16	No. 20	No. 25	No. 30	No. 35	Pan	Size (mm)	Std Screen Size			
Size (mm)	1.70	1.18	0.85	0.71	0.60	0.50	<0.50	Wt. Avg.	Mesh Opening (mm)			
Lab 0.5	0.0	0.0	0.0	0.0	0.0	0.0	100.0					
Lab 2.0	0.0	0.0	1.0	3.6	2.0	9.0	85.0					
Lab 2.4	0.1	0.4	2.1	5.7	2.4	15.0	72.8					
Hammermill Grind	Enzyme Dosage AGU's	Ethanol	Residual Carbohydrates Wt. %					Byproducts Wt. %			Total	Residual Starch %
Size (mm)	per Gram DSC	Vol. %	DP4+	DP3	Malt	Gluc	Fruc	Glyc	Lactic	Acetic	% Solids	dw
24 Hour Fermentation Results by HPLC												
0.5	1.0	13.47	0.37	0.02	ND	0.03	0.15	0.92	0.05	ND	19.6	
2.0	1.0	12.68	0.36	0.02	ND	0.03	0.08	0.94	0.05	ND	18.9	
2.4	1.0	12.71	0.37	0.02	ND	0.03	0.14	0.95	0.05	ND	18.9	
0.5	1.5	14.15	0.39	0.02	ND	0.04	0.08	0.91	0.05	ND	17.9	
2.0	1.5	13.72	0.37	0.02	ND	0.04	0.08	0.93	0.05	ND	17.8	
2.4	1.5	13.86	0.38	0.02	0.01	0.05	0.08	0.94	0.05	ND	17.3	

TABLE 4-continued

Impact of Grind Particle Size and Glucoamylase Dosage on Fermentation Efficiency												
48 Hour Fermentation Results by HPLC												
0.5	1.0	17.73	0.38	0.02	0.02	0.02	0.10	1.05	0.06	ND	14.3	
2.0	1.0	17.31	0.38	0.02	0.02	0.02	0.10	1.09	0.06	ND	14.5	
2.4	1.0	17.10	0.38	0.02	0.02	0.02	0.10	1.08	0.06	ND	15.0	
0.5	1.5	18.36	0.42	0.03	0.02	0.03	0.09	1.05	0.05	ND	13.0	
2.0	1.5	18.23	0.40	0.02	0.02	0.02	0.08	1.09	0.06	ND	13.6	
2.4	1.5	18.14	0.41	0.02	0.02	0.02	0.09	1.07	0.06	ND	13.6	
72 Hour Fermentation Results by HPLC												
0.5	1.0	18.99	0.40	0.02	0.02	0.05	0.10	1.10	0.06	ND	12.5	8.99
2.0	1.0	18.42	0.38	0.02	0.01	0.05	0.10	1.13	0.06	ND	12.8	11.34
2.4	1.0	18.54	0.39	0.02	0.02	0.05	0.10	1.14	0.06	ND	12.7	17.48
0.5	1.5	19.05	0.42	0.03	0.02	0.05	0.09	1.08	0.05	ND	11.8	6.81
2.0	1.5	18.78	0.40	0.02	0.02	0.05	0.09	1.11	0.06	ND	12.0	7.07
2.4	1.5	18.69	0.40	0.02	0.02	0.05	0.09	1.09	0.06	ND	12.2	8.72

**[0174] Results and Discussion**

**[0175]** Results are shown in Table 4, and FIGS. 3A, 3B, 3C, 3D. The data illustrates that smaller grind size provided higher ethanol yield and lower residual starch. At lower glucoamylase doses, grind size was a more influential factor. As the particle size of the grind increased, a higher enzyme dosage was required to achieve the same relative results.

**Example 5**

**Impact of Grind Particle Size, Glucoamylase Type, and Acid Fungal Amylase Dosage on Fermentation Efficiency**

**[0176]** Results of an embodiment of the method of the present invention were evaluated with varying particle size

of the ground plant material, varying glucoamylase type, and dosage of acid fungal amylase.

**[0177] Materials and Methods**

**[0178]** Whole Corn and corn flour was obtained from Dakota Ethanol LLC in Wentworth, S.D. The whole corn was ground through a 2.0 mm screen as in prior examples using a lab scale hammermill. Fermentations were set up in a similar manner as prior Examples according to the outline in Table 5.

TABLE 5

Impact of Grind Particle Size, Glucoamylase Type, and Acid Fungal Amylase Dosage on Fermentation Efficiency									
Screen Size (mm)	No. 12	No. 16	No. 20	No. 25	No. 30	No. 35	Pan	Sieve Size	
2.0 mm	0.0	0.2	1.4	3.2	3.6	15.3	<0.50	Pore Size (mm)	
Plant Hammermill #7	10.2	18.9	14.0	7.4	3.8	7.9	38.1	"Finer Grind"	
								"Coarser Grind"	
Experimental Outline for Example 5									
AFAU Dose Per Gram DSC			AGU Activity per gram DSC						
From SP 288	From GA	Total AFAU	From SP 288	From GA	Total AGU's				
SP 288	GA	Total AFAU	SP 288	GA	Total AGU's				
Units/gm	Units/gm	Units/gm	Units/gm	Units/gm	Units/gm	Flour	L-400 GA	Fermenter	
DSC	DSC	DSC	DSC	DSC	DSC	Grind	Applied	#	
0	0.20	0.20	0.00	1.10	1.10	Finer	Spirizyme+	1	
0.20	0.20	0.39	0.02	1.10	1.12	Finer	Spirizyme+	2	
0.59	0.20	0.78	0.05	1.10	1.15	Finer	Spirizyme+	3	
0.00	0.20	0.20	0.00	1.10	1.10	Coarser	Spirizyme+	4	
0.20	0.20	0.39	0.02	1.10	1.12	Coarser	Spirizyme+	5	
0.59	0.20	0.78	0.05	1.10	1.15	Coarser	Spirizyme+	6	
0.00	0.08	0.20	0.00	1.10	1.10	Finer	Distillase	7	
0.20	0.08	0.39	0.02	1.10	1.12	Finer	Distillase	8	
0.59	0.08	0.78	0.05	1.10	1.15	Finer	Distillase	9	
0.00	0.08	0.20	0.00	1.10	1.10	Coarser	Distillase	10	
0.20	0.08	0.39	0.02	1.10	1.12	Coarser	Distillase	11	
0.59	0.08	0.78	0.05	1.10	1.15	Coarser	Distillase	12	

TABLE 5-continued

Impact of Grind Particle Size, Glucoamylase Type, and Acid Fungal Amylase Dosage on Fermentation Efficiency												
72 Hour Results												
Fermenter #	% Ethanol	Residual Carbohydrates Wt. %					Byproducts Wt. %				Total	Residual
		DP4+	DP3	Malt	Gluc	Fruc	Glyc	Lactic	Acetic	% Solids	Starch % dw	
1	17.84	0.36	0.01	0.01	0.01	0.12	0.89	0.07	ND	15.31	17.09	
2	18.17	0.36	0.01	0.01	0.01	0.12	0.89	0.06	ND	15.12	16.53	
3	18.57	0.36	0.01	0.01	0.02	0.12	0.90	0.06	ND	14.72	16.31	
4	19.46	0.45	0.02	0.03	0.28	0.16	0.92	0.04	ND	14.36	15.14	
5	19.65	0.44	0.02	0.04	0.57	0.17	0.92	0.04	ND	14.49	14.97	
6	19.74	0.42	0.01	0.04	0.59	0.19	0.90	0.04	ND	14.40	13.81	
7	14.42	0.37	0.01	0.01	ND	0.05	0.65	0.16	ND	20.24	36.27	
8	15.89	0.37	0.01	0.01	ND	0.10	0.77	0.07	ND	16.68	27.24	
9	17.25	0.37	ND	0.01	0.01	0.11	0.86	0.06	ND	15.97	20.43	
10	17.19	0.46	0.01	0.01	0.01	0.10	0.80	0.05	ND	18.19	31.43	
11	18.35	0.44	0.01	0.01	0.03	0.14	0.87	0.05	ND	16.16	24.07	
12	19.30	0.42	0.01	0.01	0.06	0.15	0.92	0.05	ND	14.95	18.01	

**[0179] Results and Discussion**

**[0180]** Final fermenter results are shown in FIGS. 4A, 4B, and 4C. Conventional glucoamylase enzymes such as Distillase from Genencor International contained a very low level of acid fungal amylase activity. Spirizyme Plus contained about 2.5 times as much AFAU activity per ml of enzyme and exhibited improved performance for hydrolyzing raw starch. SP 288 acid fungal amylase contained a relatively low level of glucoamylase.

**[0181]** It was possible to gain an understanding of the importance of grind size, glucoamylase dosage level, and acid fungal amylase dosage level on fermentation performance. Improved results were obtained when a "finer" grind was combined with glucoamylase containing enhanced acid fungal amylase levels. With a coarser grind, high dosage levels of glucoamylase including acid fungal amylase yielded improved fermentation performance. Glucoamylase including acid fungal amylase provided benefits as grind size decreased.

**Example 6**

**Impact of Fermenter Dry Solids Loading and Temperature on Fermenter Kinetics and Ethanol Performance**

**[0182]** An embodiment of the present invention was employed to produce ethanol from corn. This process produced high alcohol corn beer, high protein, high fat, and high fiber distiller's dried grain. Comparison with conventional saccharification and liquefaction process indicate superior performance of the present method.

**[0183] Materials and Methods**

**[0184]** Example 6 was set up in a manner similar to prior examples except the initial fermentation solids and temperature were varied as described in the presentation of the results.

**[0185] Results**

**[0186]** An interesting feature of the present raw starch fermentation process is the ability to enhance the rate of fermentation through increasing the solids content or initial

temperature of fermentation. Solids loading, temperature, grind size, glucoamylase dosage, acid fungal amylase dosage, and yeast dosage can be combined to increase the performance of raw starch fermentation. FIGS. 5A, 5B, 5C, 5D, 5E, 5F, 5G, 5H, 5I, and 5J illustrate the influence of temperature at different solids loadings.

**[0187]** The residual starch values reported for this Example suggest that temperature can be used to improve the efficiency of raw starch fermentations at intermediate fermentation gravities, which are defined as fermentation solids levels which would yield between 15% to 18% ethanol. The fermentation temperature could be used to accelerate raw starch fermentations so that they finish in less than 48 hours, yet still achieve alcohol levels of 15% to 18%, with acceptable residual starch levels. The increased fermentation set point will help to accelerate enzymatic conversion of native starch to glucose, which appears to be the rate limiting step in the raw starch process. Fermentation performance using higher temperature set points is an aspect of the process for intermediate ethanol ranges, especially when viewed from the perspective of prior examples establishing that raw starch fermentations can tolerate a higher level of residual starch in the residual distillers dried grains and with distillers dried grains solubles, and still produce excellent quality DDG or DDGS according to the proximate analysis. Alternatively, the dry substance of raw starch fermentations can be increased by approximately 20% to increase the rate of fermentation, while producing higher alcohol content in the fermenter and more DDGS with excellent quality even if the residual starch levels are high. By balancing the above inputs, a yield versus throughput economic optimization can be done with a significant decrease in difficulty. The ease of operating a high gravity, high throughput process while producing a saleable DDGS is significantly enhanced by the raw starch process.

**Example 7**

**Advantageous Aspects of Ethanol Production by the Present Process**

**[0188]** A variety of fermentation runs were conducted and the results were evaluated and compiled to demonstrate the

increased alcohol production and production of distiller's dried grain by the present process.

#### [0189] Ethanol Production

[0190] The present method produced ethanol containing corn beer with greater than 18 vol-% ethanol. Runs produced at least 18 vol-% ethanol and up to 23 vol-% ethanol within 48 to 96 hours of incubation and fermentation. The beer contained these high levels of ethanol even when it also included higher levels of residual starch. After 24 hours of incubating and fermenting the corn beer contained 9-16.5 or 12-15 vol-% ethanol. After 48 hours of incubating and fermenting the corn beer contained 13-20 vol-% ethanol. Ethanol production was linear up to a level of 14-16 vol-%. A compilation of ethanol production results from various runs is illustrated at least in FIGS. 6A and 6B.

[0191] The beer contained approximately 0.4 to 0.5 wt-% less glycerol than conventional fermentation at otherwise identical fermentation conditions (FIG. 7). The beer contained less extracted oil from the germ fraction, resulting in reduced fouling and lower VOC emissions in the water vapor during drying of the residual animal feed product. (Table 1) The beer contained less extracted oil from the germ fraction, resulting in reduced fouling and lower CO emissions in the water vapor during drying of the residual animal feed product (Table 1). The beer contained less fusel oil (FIG. 8), which inhibits yeast cell growth and fermentation if these alcohol compounds are unintentionally recycled in distillation side stripper bottoms streams. Fusel oils are also an undesirable component of potable alcohol manufacturing operations, so the present process offers an improved method of production of potable alcohol. The beer also contained less lactic and acetic acid relative to the conventional process. The beer also contained higher yeast cell counts, which contributes to improved feed products.

[0192] In addition, the present process maintained yeast at or above 300 cells/mL in these numerous runs. Yeast budding was observed in at least 40% of the yeast from hours 0-20 of incubating and fermenting and/or at least 15-20% of the yeast after hours 60 of incubating and fermenting. These yeast counts and budding are higher than observed in the conventional process.

#### Example 8

##### The Present Process Maintains Low Levels of Glucose, Maltose (DP2), Maltotriose (DP3), and Dextrins (DP4+)

[0193] The levels of glucose, maltose (DP2), maltotriose (DP3), and dextrins (DP4+) produced by an embodiment of the present invention was compared to a conventional process. The present method exhibited decreased levels of glucose, maltose (DP2), maltotriose (DP3), and dextrins (DP4+) respectively. Comparison of the level of glucose to the conventional process indicates superior performance of the present method.

#### [0194] Materials and Methods

##### [0195] Experiment 1

[0196] Whole Corn and corn flour was obtained from Dakota Ethanol LLC in Wentworth, S.D. The whole corn for continuous ethanol fermentation examples was ground

through a 0.5 mm screen as in prior examples using a lab scale hammermill. The whole corn for SSF examples was ground through a #4 screen using a commercial scale Bliss hammermill, which achieved approximately 50% of the ground flour passing through a 0.5 mm screen as measure in a sieve test of the flour.

[0197] Batch fermentations were set up in a similar manner as Example 1. Continuous ethanol fermentation was evaluated in a bench top system consisting of a refrigerated cold slurry tank followed by five (5) fermenters operating in continuous mode and finishing with a beerwell collecting the fermented beer. The volume of each fermentation stage was approximately two (2) liters. When operated at a mash flow rate of 1.5 to 2.0 ml per minute, the average fermentation time was approximately ninety-six (96) hours. Average fermenter fill solids was approximately 30-35% dry solids corn, depending on the starch content of the substrate. The mash slurry for feeding fermentation was prepared every 3 to 4 days and maintained between 6 to 12 degrees Centigrade to discourage bacterial growth in the feed tank.

[0198] The mash preparation procedures did not sterilize the mash prior to fermentation, and the fermentation train was operated with no antibiotic addition to inhibit bacterial contaminants. The mash was stored at a cold temperature to reduce the amount of work required for substrate preparation. 15 to 20 ml of 50% urea liquor was added to the cold slurry tank, which had a final mash volume of approximately 9000 liters.

[0199] Each fermenter in the continuous series was fed from the prior fermenter, while the first fermenter was fed directly from the cold slurry tank. Fermentation temperature was held at a constant 82° F. through the five (5) stage fermentation. Glucoamylase was dosed into the first fermenter to provide a dosage of approximately 2.0 to 2.4 AGU's per gram dry substance corn. Fali yeast, obtained from Fleischmann's Yeast, was added at a rate of approximately 0.65 grams per liter of slurry makeup, and was batched into the cold slurry each time fresh mash was prepared.

##### [0200] Experiment 2

[0201] A continuous fermentation run was set up employing the procedure described above for experiment 1. Lactic acid and acetic acid measurements were taken at various times and stages during the continuous multistage fermentation process. Toward the end of the run, the initial slurry pH was purposely increased, as shown, to challenge the system microbiologically. In certain circumstances, slurry pH was intermittently lowered to keep contamination in check (see, e.g., FIGS. 16A, 16B, and 16C).

##### [0202] Experiment 3

[0203] Data in Experiment 3 was created from the continuous fermentation system examples described in Examples 1, 2, and 8. Residual starch was measured using a commercially available starch assay (the Megazyme® starch assay). This assay works for samples ranging in starch content from 0-100%, which makes it applicable for residual starch analysis as well as starch assaying in raw grain. This method is an enzymatic conversion based assay that uses alpha amylase and amyloglucosidase to convert starch to glucose. The resulting glucose is then measured via HPLC and the starch content calculated.

**[0204] Results and Discussion**

**[0205]** FIGS. 9A and 9B illustrate that the present process maintained low levels of glucose during simultaneous saccharification and fermentation (SSF) and continuous raw starch fermentations. Although not limiting to the present invention, it is believed that this low level of glucose reduces potential reactions such as reversion, condensation, or Maillard Browning Reactions. Such reactions in turn can reduce ethanol yield. The data compiled in this example demonstrates that the process maintained glucose at levels at or below 3 wt-% for the entire run and at or below 1 wt-% for about 90% of the run. In particular, the process maintained glucose at levels at or below 1 wt-% from hours 12-54 of incubating and fermenting.

**[0206]** FIGS. 10-12 illustrate that the present process maintained low levels of dextrin during SSF and continuous raw starch fermentation. FIGS. 10A and 10B illustrate that the present process maintained maltose (DP2) at levels at or below about 0.2 wt-% during simultaneous saccharifying and fermenting and below about 0.34 wt-% during continuous raw starch fermentation. The data shown in FIG. 11A demonstrate that the process maintained low levels of maltotriose (DP3) during simultaneous saccharifying and fermenting at levels at or below 0.2 wt-% and at or below 0.1 wt-%. The data shown in FIG. 11B demonstrate that the present process maintained low levels of maltotriose (DP3) during a continuous raw starch fermentation at levels at or below 0.25 wt-%.

**[0207]** The data shown in FIG. 12A demonstrate that the process maintained low levels of dextrans (DP4+) during simultaneous saccharifying and fermenting at levels at or below 1 wt-% and at or below 0.5 wt-%. The data shown in FIG. 12B demonstrate that the process maintained low levels of dextrans (DP4+) during continuous raw starch system at levels at or below 0.3 wt-%.

**[0208]** The results of experiment 2 show that initial slurry pH levels up to approximately 5.8 in the present method (FIG. 16A) resulted in acceptable ethanol yields and maintained acidic fermentation contaminants within a tolerable range (e.g., fermentation was not inhibited). The percentage of lactic acid remained less than 0.45 (in most cases less than 0.35) (FIG. 16B). The percentage of acetic acid remained less than 0.18 (in most cases less than 0.06) (FIG. 16C). This embodiment of the present method resulted in consistently low lactic and acetic acid levels and stable pH levels. This resulted in greater ethanol production, which was at least in part likely due to less yeast stress.

**[0209]** The results of experiment 3 demonstrate that a continuous embodiment of the present method produced residual starch levels lower than that produced by the conventional process (FIG. 17). The residual starch levels produced using this embodiment of the present method remained lower than the residual starch levels of the conventional process (FIG. 17). The percentage of starch produced using this embodiment of the present method remained at about twenty (e.g. 21) or less (FIG. 17) whereas the percentage starch produced using the conventional process was as high as 27 (FIG. 17).

**[0210] Discussion**

**[0211]** Although not limiting to the present invention, it is believed that as glucose is formed during fermentation, it is

quickly metabolized by the yeast, which resulted in low glucose levels. The slight increase in glucose observed at the end of fermentation suggests a drop in yeast viability. Again, not to limit the present invention, this can be explained by a decrease in yeast viability and fermentation that results in glucose production rates exceeding metabolic utilization rates (fermentation of glucose no longer keeps up with production).

**[0212]** According to an embodiment of the present invention, temperature staging can be employed to minimize residual glucose production. That is, the temperature of the fermentation can be reduced as the fermentation progresses. It is believed that, in general, for every 10° C. (18° F.) increase in temperature, the rate of an enzymatic reaction approximately doubles. In an embodiment of the present method, for example, enzyme action can be slowed by decreasing the temperature of the fermentation mixture after a time period, such as after 30 hours. It is believed that cooling also maintains yeast viability, so that fermentation can continue to utilize the glucose that has been formed. Conventional commercial variations of multistage continuous fermentation processes exist. One such conventional process includes running a saccharification stage prior to fermentation to provide fermentable glucose for a more rapid yeast fermentation. The present process does not require a saccharification stage before fermentation and produces improved results. Another conventional continuous process includes aerating the 1<sup>st</sup> fermentor, and possibly the second fermentor, to encourage yeast growth. The present process provides improved results and does not require aeration of the fermentor. Some conventional continuous systems employ a yeast recycle method. The present method does not require yeast recycling and provides improved results. This embodiment of the present invention is superior to such conventional continuous fermentation systems. The present invention can employ simultaneous saccharification and fermentation of raw starch and can operate at high gravity. In an embodiment, the process of the present invention can produce ethanol at fast rates despite the apparent lack of adequate fermentable substrate.

**[0213]** A continuous ethanol production embodiment of the present method maintained low acidity levels throughout the fermentation cycle. These experiments indicate that an embodiment of the present method employing continuous fermentation created low, manageable levels of lactic and acetic acid. Low levels of lactic and acetic acid can be advantageous for maintaining a stable pH in fermentation, and can also decrease yeast stress and increase ethanol production.

**[0214]** A continuous ethanol production embodiment of the present method maintained lower starch levels throughout the fermentation cycle. Comparison of the present residual starch level to the conventional process provides an indication of advantageous performance from the present method. The mass balance of the present raw starch process suggests that residual starches can actually be higher in this process relative to the conventional, while still achieving a higher ethanol yield and improved proximate mass balance.

## Example 9

## The Present Process Produces DDGS With Less Caking and Compacting

[0215] The DDGS according to an embodiment of the present invention was compared to that produced by a conventional process. The present method produced an inventive DDGS that exhibited less caking compared to DDGS produced by the conventional process. The present DDGS with less caking is superior to conventional DDGS.

## [0216] Materials and Methods

[0217] The DDGS was collected as a co-product of ethanol production from the conventional high temperature liquefaction process and from the process of the present invention. The caking/collapse assay was performed by filling a 500 ml cylinder with approximately 400 ml of DDGS. Attention was given to avoiding physical packing of the DDGS when filling the cylinder. After filling, a 4.4 cm diameter disc weighing 78 grams was placed on top of the DDGS, followed by placement of 1.5 kg of lead shot (in an appropriately sized plastic bag) on top of the disc. Assay preparation was completed by covering each cylinder with a plastic bag and sealing the apparatus with a rubber band to prevent moisture loss. The weight applied to the DDGS is used to exaggerate the effect and approximate the conditions which DDGS is exposed to during transport, for example, in a railcar. The level of the DDGS is noted at the beginning of storage and at various times during storage at a temperature of 50° C. The measured height of the collapsed (caked) DDGS was compared to the initial height of the DDGS. The measured height was compared to the initial height as an estimate of the tendency of the product to collapse or cake.

## [0218] Results

[0219] The DDGS from the present invention shows less caking collapse over time (FIG. 13) when compared to the DDGS of the conventional process. Over a twenty-five hour compaction time the DDGS according to the present invention collapsed only 4-5% of the initial volume as compared to 10-14% of the volume collapse for DDGS of the conventional process.

## [0220] Discussion

[0221] The compaction of DDGS at controlled conditions models the DDGS caking observed in the containers of transportation vehicles, for example railcars and trucks. DDGS produced using the process of this invention exhibited less caking related collapse than that of the conventional process, indicating superior performance of the present method.

[0222] Although not limiting to the present invention, it is believed that the observed compaction is consistent with that suggested by glass transition theory. For example, glass transition temperature increases with molecular weight for polymers such as those found in DDGS. The present DDG includes higher levels of such polymers and should exhibit a higher glass transition temperature. It is believed that product moisture, storage temperature, and chemical composition can impact the transition of DDGS from an amorphous glass to an amorphous rubber phase. DDGS in the rubber phase compacts more readily than DDGS in the glass phase.

## Example 10

## The Present Process can Employ High Protein Corn to Produce High Protein DDGS and High Levels of Ethanol

[0223] In an embodiment, the present invention can include fermenting high protein corn to produce high protein DDGS and high levels of ethanol. This provides for advantageous flexibility for processing high protein corn.

## [0224] Materials and Methods

[0225] DDGS was collected as a co-product of ethanol production from fermentation of various corn hybrids with fermentations set up in a similar manner as Example 1. All fermentations were set up using identical conditions. Different corn hybrids were tested using various grind sizes using a lab scale hammermill. The hammermill screen size was varied from 0.5 mm to 4.0 mm to create flour particle sizes ranging from fine (0.5 mm screen) to coarse (4.0 mm screen).

## [0226] Results

[0227] FIG. 15A illustrates the dependence of protein level in DDGS on grind size. This figure illustrates the inverse correlation between grind size and protein: as particle size increases the protein content of DDGS decreases for each tested corn hybrid (FIG. 15A). FIG. 15B illustrates the dependence of starch level in DDGS on grind size. This figure illustrates a positive correlation between grind size and starch content in: as particle size increases the starch content of the DDGS increases for each tested corn hybrid (FIG. 15B). FIG. 15C illustrates the dependence of ethanol production on grind size. This figure illustrates that as particle size decreases there is an increase in ethanol production (FIG. 15C).

## [0228] Discussion

[0229] Reduced particle size arising from grinding of the corn enables higher ethanol yields and higher protein DDGS to be created. A strong correlation is also seen between the initial protein content of the corn and the resulting protein content of the DDGS. In the conventional process, higher protein corn is undesirable because it lowers fermentable starch content. The conventional process, being more constrained by viscosity arising from liquefaction, limits the processor's ability to maintain fermentables by increasing the solids level in fermentation. The present method is less constrained by viscosity, such that fermentable solids can be increased to maintain potential ethanol production titers while simultaneously producing a higher protein DDGS. The higher protein DDGS can be used for any of a variety of purposes.

[0230] It should be noted that there is significant effort within the current industry to encourage the use of "highly fermentable corn" hybrids. The "highly fermentable corn" hybrids can have a higher starch concentration and not a high protein concentration. This example demonstrates that higher protein corn hybrid varieties of standard #2 yellow corn can be used to obtain high levels of ethanol production. Despite standard #2 yellow corn lower starch contents, fermenter dry solids can be increased to maintain ethanol % levels in the fermenter while producing a higher protein DDGS.

## Example 11

The Raw Starch Process Enables Production of  
Co-Product With Inventive Features

[0231] In an embodiment, the present invention provides improved access to the prolamin protein (such as zein) fraction of cereal grains. The high protein content of DDG and DDGS is useful in compounding.

## [0232] Results and Discussion

[0233] This results in DDG/DDGS with varying ratios of prolamin protein (such as zein) and residual starch. FIGS. 14A and 14B show the relationship of: wet cake, syrup starch, and protein levels. As the residual starch in the wet cake reduces the protein in the wet cake increases. This indicates an inverse relationship. A similar response occurs in the syrup fraction.

[0234] It should be noted that, as used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to a composition containing "a compound" includes a mixture of two or more compounds. It should also be noted that the term "or" is generally employed in its sense including "and/or" unless the content clearly dictates otherwise.

[0235] All publications and patent applications in this specification are indicative of the level of ordinary skill in the art to which this invention pertains.

[0236] The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention.

## We claim:

1. A process for producing ethanol from plant material, comprising:

reducing the plant material to produce material comprising starch;

the reduced plant material have particle size such that at least about 50% of the particles fit through a sieve with a 0.1-0.5 mm mesh;

saccharifying the starch, without cooking, with an enzyme composition;

fermenting the incubated starch to yield a composition comprising at least 15 vol-% ethanol;

fermenting comprising reducing temperature of fermenting mixture; and

recovering the ethanol and co-products from the fermentation

2. The process of claim 1, wherein plant material comprises corn, which comprises high amylopectin starch.

3. The process of claim 1, wherein the plant material comprises corn, sorghum, millet, wheat, barley, rye, or mixtures thereof.

4. The process of claim 3, wherein the corn comprises waxy corn.

5. The process of claim 3, wherein the corn comprises high protein corn.

6. The process of claim 3, wherein the corn comprises #2 yellow dent corn.

7. The process of claim 1, comprising reducing the plant material with hammer mill, roller mill, or both hammer mill and roller mill.

8. The process of claim 7, comprising reducing the plant material to produce plant material of a size that at least 35% of the reduced plant material fits through a 0.1-0.5 mm screen.

9. The process of claim 1, comprising reducing the plant material with particle size reduction emulsion technology.

10. The process of claim 1, comprising simultaneous saccharifying and fermenting.

11. The process of claim 1, comprising decreasing temperature during saccharifying, fermenting, or simultaneous saccharifying and fermenting.

12. The process of claim 1, comprising saccharifying, fermenting, or simultaneous saccharifying and fermenting at temperature of 25-40° C.

13. The process of claim 1, comprising saccharifying, fermenting, or simultaneous saccharifying and fermenting at temperature of 27-35° C.

14. The process of claim 1, comprising reducing temperature from about 40° C. and to about 25° C. during saccharifying, fermenting, or simultaneous saccharifying and fermenting.

15. The process of claim 1, comprising saccharifying, fermenting, or simultaneous saccharifying and fermenting at pH of about 3.0 to about 6.0.

16. The process of claim 1, comprising saccharifying, fermenting, or simultaneous saccharifying and fermenting at pH of about 4.1 to about 5.3.

17. The process of claim 1, comprising a pH of about 4 to about 4.5 at start of fermentation fill.

18. The process of claim 1, comprising a pH of about 5 to about 5.5 as ethanol production reaches maximum level.

19. The process of claim 1, comprising increasing pH from about 4 to about 5.3 during saccharifying, fermenting, or simultaneous saccharifying and fermenting.

20. The process of claim 1, comprising decreasing solid content from about 40% to about 15% during saccharifying, fermenting, or simultaneous saccharifying and fermenting.

21. The process of claim 1, wherein the enzyme composition comprises alpha amylase, glucoamylase, protease, or mixtures thereof.

22. The process of claim 1, wherein saccharifying, fermenting, or simultaneous saccharifying and fermenting comprises adding protease.

23. The process of claim 1, wherein saccharifying, fermenting, or simultaneous saccharifying and fermenting comprises adding backset.

24. The process of claim 1, wherein saccharifying, fermenting, or simultaneous saccharifying and fermenting comprising adding nitrogen.

25. The process of claim 1, comprising saccharifying and fermenting at rates that maintain concentration of glucose less than 3 wt-% in fermentation.

26. The process of claim 1, comprising saccharifying, fermenting, or both saccharifying and fermenting with about 0.1 to about 10 acid fungal amylase units (AFU) per gram of dry solids reduced plant material and about 0.1 to about 6 glucoamylase units (AGU) per gram dry solids reduced plant material.

27. The process of claim 1, comprising starting saccharifying, fermenting, or both saccharifying and fermenting with about 25 to about 45 wt-% reduced plant material in water.

28. The process of claim 1, comprising starting saccharifying, fermenting, or both saccharifying and fermenting with residual starch at up to 20%.

29. The process of claim 1, comprising producing greater than 18 vol-% ethanol in about 48 to 96 hours.

30. The process of claim 1, comprising producing 18 vol-% to about 23 vol-% ethanol.

31. The process of claim 1, further comprising recovering the solids from the fermentation.

32. The process of claim 31, recovering before, during, and after recovering the ethanol.

33. The process of claim 31, comprising recovering distiller's dried grain.

34. The process of claim 31, wherein the distiller's dried grain comprises about 30-38 wt-% protein, about 11-19 wt-% fat, about 25-37 wt-% fiber.

35. The process of claim 31, wherein the distiller's dried grain comprises at least about 30% protein.

36. The process of claim 1, comprising running the process as a batch process or as a continuous process.

37. A process of drying distillation products from the production of ethanol, comprising:

producing starch from corn and ethanol from the starch; producing reduced stack emissions of 1.47 or less pounds of volatile organic compounds per ton of corn.

38. The process of 37, further producing reduced stack emissions of 0.98 or less pounds of carbon monoxide per ton of corn processed.

39. A process for producing ethanol from plant material, comprising:

reducing the plant material to produce material comprising starch;

saccharifying the starch, without cooking, with an enzyme composition comprising acid fungal amylase;

fermenting the incubated starch to yield a composition comprising at least about 18 vol-% ethanol;

recovering ethanol from the fermentation.

40. A distiller's dried grain comprising at least about 30 wt-% protein.

41. A distillers dried grain comprising about 30-38 wt-% protein, about 11-19 wt-% fat, about 25-37 wt-% fiber.

42. A corn beer comprising at least about 18% ethanol.

\* \* \* \* \*

# United States Patent [19]

Yamamoto et al.

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## [54] PROCESS FOR SACCHARIFICATION OF ROOTSTOCKS AND SUBSEQUENT ALCOHOL FERMENTATION

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### [57] ABSTRACT

For saving the heat energy required for alcohol production, processes are provided for subjecting rootstocks (Rhizomes) to enzymatic hydrolysis without conventional cooking with steam in order to obtain a high concentration saccharified material which is then subjected to alcohol fermentation.

The first process comprises dipping raw rootstocks in a dilute acid for the purpose of sterilization, then crushing the sterilized rootstocks, macerating the crushed rootstocks, dextrinizing the macerated material by the action of bacterial  $\alpha$ -amylase and adding to the dextrinized material glucoamylase and yeast for alcohol fermentation to effect saccharification thereof and subsequent alcohol fermentation.

The second process comprises sterilizing rootstocks with a dilute acid and then crushing it, followed by adding blended maceration enzymes to effect maceration and saccharification with subsequent alcohol fermentation by yeast.

34 Claims, No Drawings

## PROCESS FOR SACCHARIFICATION OF ROOTSTOCKS AND SUBSEQUENT ALCOHOL FERMENTATION

### BACKGROUND OF THE INVENTION

#### 1. Field of the Invention

This invention relates to a process for saccharification of rootstocks and subsequent alcohol fermentation. More particularly it relates to a process for saccharifying rootstocks such as sweet potatoes, cassava roots and white potatoes according to an enzymatic processing without conventional steaming, and using the resulting material as a feed substrate for alcohol fermentation.

#### 2. Description of the Prior Art

Fossil energy resources have been getting scarcer, and as a countermeasure to this, conversion of renewable resources i.e. biomass into energy resources and utilization of biomass as chemical raw materials have recently come to public notice.

Heretofore, according to conventional processes for saccharification of rootstocks such as sweet potatoes, Cassava roots, white potatoes, etc. and subsequent alcohol fermentation, such raw materials have been once cooked for gelatinization of starch followed by adding  $\alpha$ -amylase to act on the gelatinized starch to convert it into dextrin, which, in turn, has been subjected to alcohol fermentation.

However, such conventional processes have had drawbacks in that large scale equipments such as high pressure cookers have been required and also a large quantity of heat has been needed for cooking. In particular, such a quantity of heat and that required for distilling off ethanol from the fermented beer for alcohol has increased the amount of heat required for the overall process of ethanol fermentation, whereby no commercially effective process could have been achieved. For example, conventional cooking of rootstocks to gelatinize starch for saccharification proceeding to subsequent alcohol fermentation has been carried out under pressure of about 2.5 kg/cm<sup>2</sup> for about 30 to 60 minutes and the quantity of steam required for the heating has amounted to about 30% of the total quantity of steam required for alcohol production. Further, drain mixes in the material during the cooking treatment, whereby the feed substrate for alcohol is diluted to reduce the concentration of the alcohol obtained by fermentation. Furthermore, a quantity of heat required for alcohol distillation carried out in the final step is increased and the yield of the product alcohol per unit of a quantity of heat is reduced. According to the conventional processes, the heat energy required for the alcohol distillation has amounted to as large as  $\frac{1}{2}$  of the combustion energy of the alcohol.

### SUMMARY OF THE INVENTION

An object of the present invention is to provide a process of subjecting rootstocks to enzymolysis without conventional cooking, to obtain a high concentration of a saccharified material, which is then subjected to alcohol fermentation at high concentration.

Another object of the present invention is to provide a process for saving the heat energy required for alcohol distillation.

Other objects of the present invention will be apparent from the following description.

In order to attain the above-mentioned objects, the present inventors first have noted that the object of

conventional cooking of rootstocks has consisted not only in gelatinization of starch, but in sterilization of rootstocks so that contamination by microbes should not occur during alcohol fermentation, and have made studies on a process of carrying out the sterilization of raw material rootstocks separately from the gelatinization and saccharification. As a result, it has been found that if rootstocks are washed with water to remove attached soil and immersed in a dilute acid at room temperature for several hours, it is possible to sterilize microorganisms which are harmful for alcohol fermentation. Further, they have made studies on saccharification of starch, and found that if maceration enzymes are first applied to the crushed rootstocks to form a slurry, and liquefying  $\alpha$ -amylase is then added to the slurry followed by slightly warming for dextrinization of starch, saccharification by glucoamylase is notably enhanced, and that particularly when white potatoes are used as raw material and a portion of the whole of the supernatant liquid of the macerated material formed by the action of maceration enzymes is removed, followed by the treatment with a bacterial  $\alpha$ -amylase, then a system of high concentration of alcohol fermentation can be achieved.

The first process of the present invention has been completed based on the above-mentioned findings, which process comprises:

Sterilizing rootstocks through immersion treatment in a dilute acid,

Crushing the sterilized rootstocks,

Macerating the crushed rootstock to slurry by enzymes.

Adding bacterial  $\alpha$ -amylase to the slurry followed by warming to dextrinize the starch.

Adding to the dextrinized material glucoamylase and yeast for alcohol fermentation to effect alcohol fermentation in parallel with saccharification, thereby producing a high concentration of alcohol at high rate.

The second process of the present invention comprises sterilizing rootstocks through immersion treatment in a dilute acid and then crushing them as in the first process, followed by macerating the tissue of the rootstocks by blended maceration enzymes and saccharifying starch contained in the macerated material by the action of glucoamylase, and subjecting the resulting material to alcohol fermentation.

In the first process of the present invention, since the dextrinization process is employed that is carried out at relatively high temperature such as 80°-90° C. adding bacterial  $\alpha$ -amylase, the whole reaction becomes rapid. In the second process, which is carried out without dextrinization and under a mild temperature conditions in the range of 20°-45° C. using the blended enzymes for maceration of the tissue of the root stocks and glucoamylase to saccharify raw starch, the reaction is not so rapid, but the energy for temperature elevation as needed in the case of the first process is saved.

### DESCRIPTION OF THE PREFERRED EMBODIMENTS

In the processes of the present invention, rootstocks used as a raw material are preferably selected from sweet potatoes, cassava roots and white potatoes which are available in a large amount. Such root-stocks are washed to remove soil and sand attached thereto, followed by immersing them in a dilute acid solution. As for the acid, not only mineral acids such as hydrochloric

acid, sulfuric acid, nitric acid, but organic acids such as acetic acid may be used. The concentration of the acid is preferably in the range of 0.02 to 0.08N (Normality) in the case of mineral acids, and 0.04 to 0.10N in the case of acetic acid. The pH of the acid solution is preferably in the range of 1.6 to 2.8. The immersion time required is about 4 hours or longer, preferably 6 hours or longer, at room temperature. That acid treatment makes it possible to sterilize the microbes adhered to rootstocks, which are harmful to alcohol fermentation.

Unless the above-mentioned acid immersion treatment is carried out, an unfavorable acid fermentation such as a lactic acid fermentation is liable to occur due to bacteria adhered to rootstocks. For example, when sweet potatoes were washed only with water without acid immersion, crushed and just thereafter subjected to the action of maceration enzymes, followed by adding bacterial  $\alpha$ -amylase to the macerated material to dextrinize the starch contained therein, adding saccharogenic enzyme and yeast for alcohol fermentation, and subjecting the mixture to fermentation, then lactic acid fermentation due to the action of contaminated microbes occurred and considerably retarded alcohol fermentation. Further, in another example, not only lactic acid fermentation but butyric acid fermentation occurred, which notably reduced the yield of alcohol.

Crushing of rootstocks is carried out in order to make it easily subject to the action of maceration enzymes. It may be carried out by means of such a machine as a disposer, a mincer, a slicer, a dispersion mill, etc. The crushing treatment is preferably carried out as sterilely as possible. Rootstocks are preferably crushed into as fine particles as possible, for example, those of about 8 to 20 meshes on the average. The maceration enzymes may be added either when rootstocks are crushed or thereafter.

The maceration enzymes are composed mainly of polygalacturonase such as pectin endopolygalacturonase and contain small amounts of carboxymethylcellulase, arabinoxylanase and arabinogalactanase. As for commercially available products of such enzymes, "Cellulosin AC" and "Orienzyme B" (tradenames of products made by Ueda Chemical Industrial Co., Ltd., Japan) are mentioned.

In place of the enzyme agents for macerating the crushed rootstocks, Koji of molds producing the maceration enzymes may be used. Such Koji contains mainly pectic enzymes containing a large amount of pectin endopolygalacturonase. Its amount to be added is preferably 50 to 1,000 units in terms of pectin endopolygalacturonase per 100 g of rootstocks.

The crushed rootstocks in a suitable reaction vessel are incubated with the maceration enzymes at pH of 3.5 to 4.5, at a temperature of 20° to 45° C. for 0.5 to 1 hour. The maceration reaction may be carried out either batchwisely or continuously. The amount of the maceration enzymes or Koji of molds to be added may be 50 to 1,000 units per 100 g of rootstocks in the case of sweet potatoes, and 1/5 to 1/10 of the above-mentioned units in the case of white potatoes or cassava roots.

Bacterial  $\alpha$ -amylase is then added to the macerated material obtained by the macerating reaction in order to dextrinize the starch contained in the material. This step is carried out by adjusting the pH of the macerated material to about 5 or higher, and then heating it at temperature of 80° to 90° C., and for about 15 minutes in the case of 80° C. and 1 to 3 minutes in the case of 90° C. The amount of the bacterial  $\alpha$ -amylase for this pro-

cessing may be 4 to 16 units per 1 g of starch in terms of starch liquefying activity, and the extent of the dextrinization is preferably between 5 and 12% in terms of the reducing sugar forming degree. The dextrinization reaction may be carried out either batchwisely or continuously. The addition step of the bacterial  $\alpha$ -amylase is necessary in the case of white potatoes as a raw material, but it is not always necessary in the case of cassava roots or sweet potatoes unless the high rate fermentation is desired. In addition, in the case of white potatoes which starch content is low, if the supernatant liquid of the macerated material formed by the action of the macerating enzymes is removed before dextrinization by the bacteria  $\alpha$ -amylase, it is possible to obtain such a higher concentration of alcohol as elevated as much as that corresponding to the amount of the supernatant liquid removed, in the subsequent alcohol fermentation.

The resulting liquid formed by the dextrinization is cooled and then subjected to alcohol fermentation in a conventional manner. Namely, to the dextrinized material are added saccharifying amylase (glucoamylase), yeast seed for alcohol fermentation and if necessary nutrients for the yeast depending on the kind of rootstocks, followed by fermentation under conditions of e.g. 25° C. and pH of 4.5. The saccharifying enzyme may be commercially available, liquid or powdery glucoamylase, and its amount to be added may be 2 to 10 units per 1 g of starch contained in the raw material. The fermenting yeast may be any yeast for alcohol fermentation, for Sake making or for bread baking. Further, it is also possible to use a yeast of *Pichia* genus effecting alcohol fermentation even at 30° C. or higher. In the alcohol fermentation, it is preferable to add small amounts of ammonium sulfate, potassium dihydrogen phosphate, calcium chloride, magnesium sulfate, etc. as nutrients for the yeast.

The blended maceration enzyme agent used in the second process contains pectin endopolygalacturonase, cellulase, hemicellulase, glycoamylase and acidically active  $\alpha$ -amylase. The contents of the respective enzymes in the enzyme agent has no particular limitation, but it preferably consists of 300 units or more of carboxymethylcellulase (CMC) as the cellulase per 1 kg of rootstocks (one unit: an enzymatic activity by which reducing sugar corresponding to 1  $\mu$  mole of glucose is freed at 40° C., at pH 4.2 in one minute when CMC is used as a substrate); 100 units or more of arabinoxylanase as the hemicellulase (one unit: an enzymatic activity by which reducing sugar corresponding to 1  $\mu$  mole of xylose is formed at 40° C. in one minute when arabinoxylan of rice straw is used as a substrate); and 100 units or more of arabinogalactanase (one unit: an enzymatic activity by which reducing sugar corresponding to 1  $\mu$  mole of galactose is formed at 40° C., pH 5 in one minute when arabinogalactanase of soybean seed coat is used as a substrate); 400 units or more of polygalacturonase in terms of a pectin viscosity reduction activity (one unit: an enzymatic activity by which, when the polygalacturonase is incubated with 10 ml of a 1.0% citrous pectin solution, the viscosity is reduced down to its half at 40° C., at pH 3.6 in 10 minutes); 1,500 units or more of glucoamylase in terms of a starch saccharifying activity (one unit 1  $\mu$  mole of glucose is formed at 40° C., at pH 5 in one minute); and 20 units or more of acidically active  $\alpha$ -amylase in terms of a starchdextrinizing activity determined by iodometry (one unit: an enzyme activity by which, when the  $\alpha$ -amylase is incubated with 10 ml of a 1% soluble starch at pH 4.0, its color is

reduced down to a half at 40° C. in one minute). As for the amounts of the above-mentioned respective enzymes used, although considerably larger amounts than those of the above lower limits may be employed without any obstacles, excess amounts are uneconomical; hence usually the respective upper limits are preferred to be about 6,000 units of cellulase, about 4,000 units of hemicellulase (about 2,000 units of arabinogalactanase and about 2,000 units of arabinoxylanase), about 8,000 units of polygalacturonase, about 30,000 units of glucoamylase and about 400 units of acidically active  $\alpha$ -amylase. The above blended enzyme agents may be usually prepared by adequately blending individual commercially available enzymes, and the respective enzymes may be preferably those of microorganism origin, such as cellulase, hemicellulase, polygalacturonase and acidically active  $\alpha$ -amylase, produced by fungi belonging to *Aspergillus* genus, such as *Aspergillus niger*, glucoamylase produced by fungi belonging to *Aspergillus* genus such as *Aspergillus niger* or *Rhizopus* genus, etc. They may be those produced by molds related to the above species and in the form of liquid enzyme agents. In particular, since blended enzyme agents which have so far been known as vegetable tissue degrading enzymes (maceration enzymes) contain cellulase, hemicellulase, polygalacturonase and acidically active  $\alpha$ -amylase almost similar to those in the blended enzyme agents used in the present invention it is possible to prepare suitable blended enzyme agents by blending glucoamylase to the above maceration enzymes and if necessary, further adding insufficient enzymes such as hemicellulase, pectinase, etc.

The maceration and saccharification treatment of rootstocks by the above blended enzyme agents is preferably carried out by contacting the sterilized rootstocks with the blended enzyme agents at pH of 3.5 to 5.0 and at temperature of 20° to 45° C., preferably of 20° to 35° C. Control of the pH is not always necessary, since sterilization of rootstocks with acid usually brings about the above mentioned pH range, but, if necessary, suitable mineral acid or alkali e.g. sodium hydroxide may be added for pH control in the treatment. The above temperature condition is the optimum one (up to about 55° C.) for enzymes, and within the above range, higher temperature can afford more shortened reaction time. Further, the above contact is sufficiently carried out merely by feeding crushed rootstocks together with the blended enzyme agents into a suitable vessel provided with or without stirring means. The reaction in the vessel may be carried out either batchwisely or continuously, and in the case of continuous operation, it is suitable to carry out the operation by adding water so as to give a dilution of about 0.1 to 0.5 to thereby facilitate continuous feeding of raw material and continuous recovery of products.

Thus the above contact makes it possible to saccharify substantially all of the starch contained in the raw materials for 7 days (168 hours), for example, in the case without application of bacterial  $\alpha$ -amylase of the second process, and for 36 hours, for example, in the case of the first process where  $\alpha$ -amylase is applied for dextrinizing starch. The period of time required for the saccharification varies depending on the kind of raw materials to be used, the blending ratio of the enzyme agents and their amounts used, and the reaction conditions (temperatures, pH, etc.), but, since the saccharification of starch by the enzyme agents also proceeds in the subsequent alcohol fermentation step, it may be sufficient if the above

saccharification reaction through the contact proceeds so as to effect about 50% of the theoretically calculated value of reducing activity corresponding to that of glucose.

While the macerated and saccharified material obtained in the saccharification step may be utilized as sugars for various food industries, it is particularly suitable as a raw material for alcohol fermentation such as ethanol or propanol fermentation. Alcohol fermentation by the use of the saccharified material as a raw material may be carried out in a conventional manner, that is, by adding a yeast seed for alcohol fermentation and inorganic nutrients for the yeast to the saccharified material and maintaining the mixture under suitable temperature and pH conditions for a few days to effect a good fermentation. The resulting fermented beer is then subjected to distillation.

The above alcohol fermentation step can be carried together with the above maceration and saccharification steps. In this case, the above blended enzyme agents and yeast seed for alcohol fermentation together with inorganic nutrients therefore can be at the same time added to the crushed raw material to thereby carry out the maceration of the tissue, the saccharification of the starch and at the same time, the fermentation of the resulting sugars by the yeast, within a single system.

According to the processes of the present invention, root-stocks are washed and sterilized with a dilute acid, followed by applying specified enzyme agents to the resulting material without any cooking process, whereby it is possible to effect the maceration and saccharification easily and yet with a high yield of glucose. Further, the saccharified material is subjected to alcohol fermentation whereby it is possible to produce a high concentration of alcohol and economize the energy required for the alcohol production. The above effects are considered to be due to the fact that the raw material is sterilized by a dilute acid in place of conventional cooking, and specified enzyme agents decompose cementing substances such as pectic substances and some cellulosic and hemicellulosic materials surrounding starch granules in the potato tissue, whereby substantially all of the starch particles are very easily saccharified by the action of glucoamylase.

The processes of the present invention will be concretely mentioned below by way of Examples. Examples 1 and 2 are directed to the above first process, and Examples 3 to 6 are directed to the above second process.

#### EXAMPLE 1

Two kg of sweet potato washed with water in advance were immersed in a sulfuric acid solution at pH 1.8 for 6 hours and then crushed by means of a household disposer (pH: 4.2), followed by adding commercially available maceration enzymes 1 g (containing per 1 g, 1,440 units of pectin endopolygalacturonase, 990 units of carboxy methyl cellulase, 390 units of arabinoxylanase, and 315 units of arabinogalactanase), and reaction in a 2 l capacity stainless steel vessel with stirrings sometimes at 45° C. for one hour to obtain a macerated material. Sodium hydroxide was then added to the macerated material to adjust the pH to 5.6. A bacterial  $\alpha$ -amylase preparation (3,500 units/g) (0.5 g) was then added followed by dextrinization reaction in a 1 l capacity stainless steel separable flask, while continuously injecting the mixture by means of a roller pump and continuously discharging the resulting product, under

conditions of a retention time of 10 minutes and 85° C. As a result, the starch (about 28% by weight) contained in the crushed material was dextrinized (percentage dextrinization: 11%), and the concentration of the dextrinized material was nearly 31.0% in terms of glucose after saccharification by glucoamylase. This means that the raw material was never diluted during the dextrinization and saccharification process; hence it is possible to subject the resulting material as it is to the subsequent alcohol fermentation.

To the dextrinized material (liquid) were added per 1 kg, 12.5 g of a yeast seed for alcohol fermentation (*SACCHAROMYCES CEREVISIAE* HANSEN RASSE XII), and as mineral nutrients for the yeast, 1.1 g of potassium dihydrogen phosphate, 1.5 g of ammonium sulfate, 0.13 g of magnesium sulfate and 0.13 g of calcium chloride, and 1 g of glucoamylase (1,200 units of active glucoamylase per 1 g thereof), followed by fermentation in a small type fermenter (5 l capacity) at pH 4.5 and 25° C. As a result, the alcohol concentration amounted to 16.9% by volume in 43 hours; thus a high concentration alcohol fermentation was achieved in a short time.

#### EXAMPLE 2

Two kg of washed white potato were immersed in a sulfuric acid solution at pH 1.8 for 6 hours and then crushed by means of a disposer, followed by adding 0.5 g of the same maceration enzymes as used in Example 1.

The mixture was placed in a 2 l capacity stainless steel vessel and its pH was adjusted to 4.5 with stirring, followed by keeping it at 45° C. for one hour to obtain a slurry material, which was then subjected to solid-liquid separation by means of a centrifugal decanter. Two thirds of the resulting supernatant liquid (about 520 ml from 1 kg of potato) was removed. Sodium hydroxide was then added to the residue with stirring to adjust the pH to 5.6, followed by adding 0.5 g of the same bacterial  $\alpha$ -amylase as used in Example 1 to the mixture and subjecting it to dextrinization reaction in a 1 l capacity stainless steel separable flask, while repeating on operation of continuously injecting the mixture by means of a roller pump and continuously discharging the resulting material, under conditions of a retention time of 5 minutes and 88° C. As a result, the percentage dextrinization relative to the raw material was 12%, and the total sugar content of the dextrinized material was 32%; thus it was possible to prepare a dextrinized liquid which was more concentrated by about 60% than that obtained by cooking potato followed by dextrinization by the bacterial  $\alpha$ -amylase.

To the dextrinization liquid thus prepared were then added, as in Example 1, per 1 kg of the liquid, 12 g of a yeast seed for alcohol fermentation (*S. CEREVISIAE* R. XII), 1 g of glucoamylase, and as mineral nutrients, 1.1 g of potassium dihydrogen phosphate, 1.5 g of ammonium sulfate, 0.13 g of magnesium sulfate and 0.13 g of calcium chloride, followed by subjecting the mixture to fermentation in a small type fermenter at pH 4.5 and 25° C. The resulting alcohol concentration amounted to 16.7% by volume in 44 hours; thus it was possible to achieve a high concentration alcohol fermentation which had been impossible according to the conventional process, by the use of white potato as raw material and in a very short time.

Even when raw cassava was used as raw material, almost the same results were obtained.

#### EXAMPLE 3

One kg of sweet potato as raw material was immersed in a dilute hydrochloric acid at pH 1.8 for 4 hours and just thereafter crushed and triturated by means of a disposer, followed by adding 0.05% by weight based on the weight of the raw material, of maceration enzyme agent (containing per 1 g thereof, 990 units of carboxy methyl cellulase, 315 units of arabinogalactanase, 390 units of arabinoxylanase, 1,440 units of pectin endopolygalacturonase and 69 units of acidically active  $\alpha$ -amylase) and 0.1% by weight of glucoamylase which is of *Rhizopus* origin and containing 2,510 units of glucoamylase per 1 g), and then subjecting the mixture to reaction in a 2 l capacity stainless steel vessel with stirring at pH 4.8 and 40° C. As a result, the triturated material of the raw sweet potato was decomposed to become a slurry in a few hours, and when the mixture was further incubated under the same conditions, saccharification of the starch proceeded in three days ultimately to reach the reducing sugar formation corresponding to the hydrolysis degree of 50% or more of the starch.

To this mixture but immediately after adding glucoamylase were added a yeast seed for alcohol fermentation (0.12 g as dry weight) and as mineral nutrients for the yeast, a mixture consisting of 0.25 g of potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ), 0.6 g of ammonium sulfate ( $(\text{NH}_4)_2\text{SO}_4$ ) and 0.07 g of magnesium sulfate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ) (pH: 4.0), and the resulting mixture was kept at 23° C. for 6 days, followed by distilling the whole and then determining ethanol volume in the resulting distillate, to give an ethanol yield per 1 kg of the raw material (sweet potato), of 146 ml in terms of anhydrous ethanol. This yield is similar to that in the case where the same raw sweet potato was conventionally cooked and then saccharified, followed by alcohol fermentation, but when it is taken into consideration that the consumption of the energy required for the above cooking (about 30% of the total energy required for ethanol production) was saved, it can be seen that the present invention is practically valuable.

Further, the above procedure was repeated except that the raw sweet potato as raw material was replaced by raw cassava, to give almost the same results.

#### EXAMPLE 4

One kg of raw sweet potato was subjected to acid treatment and then crushed and triturated as in Example 3, followed by adding 0.05% by weight of commercially available maceration enzymes A (containing per 1 g, 980 units of cellulase, 330 units of arabinogalactanase, 360 units of arabinoxylanase, 280 units of pectin endopolygalacturonase and 65 units of an acidically active  $\alpha$ -amylase), 580 units of pectin endopolygalacturonase isolated from the maceration enzyme as used in Example 3, per 1 kg of raw sweet potato, and 0.1% by weight of glucoamylase (of *Rhizopus* origin and containing 2,510 units of glucoamylase per 1 g thereof), and then subjecting the mixture of alcohol fermentation by adding a yeast seed. As a result, the ethanol yield in terms of anhydrous ethanol after fermentation for 7 days and distillation was 144 ml per 1 kg of raw material sweet potato.

When the same procedure as the above was repeated except that pectin endopolygalacturonase (580 units/kg raw roots) was not added, the ethanol yield in terms of

anhydrous ethanol after alcohol fermentation was 101 ml per 1 kg of sweet potato.

#### EXAMPLE 5

In place of maceration enzymes of Example 3, using 0.05% by weight of commercially available maceration enzymes B (containing per 1 kg, 1,000 units of cellulase, 75 units of arabinogalactanase, 85 units of arabinoxylanase, 2,100 units of pectin endopolygalacturonase and 90 units of an acidically active  $\alpha$ -amylase) to which was further added arabinogalactanase (120 units) and arabinoxylanase (152 units) both separated from the former maceration enzymes (these units being lesser than those in the former maceration enzymes), decomposition through maceration and saccharification, and subsequent alcohol fermentation as in Example 3 were carried out. As a result, the ethanol yield (as an anhydrous ethanol) after fermentation and distillation was 145 ml per 1 kg of raw sweet potato.

In addition, when the above enzymes B alone were used (that is, arabinogalactanase and arabinoxylanase were not further added), the ethanol yield per 1 kg of sweet potato was 105 ml.

#### EXAMPLE 6

Ten kg of raw sweet potato were immersed in a hydrochloric acid solution and then crushed and triturated by means of a disposer in the same manner as in Example 3, followed by adding a mixture consisting of 0.05% of the same maceration enzymes as in Example 3, 1.2 g of a yeast for alcohol fermentation based on the dry weight thereof, 2.5 g of potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ), 6 g of ammonium sulfate ( $(\text{NH}_4)_2\text{SO}_4$ ) and 0.7 g of magnesium sulfate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ) as mineral nutrients (pH: 4.6), and then keeping the mixture at 15° to 25° C. for 5 days, after which the whole of the resulting fermented beer was subjected to distillation and the ethanol quantity in the distillate was determined, to give an ethanol yield (as an anhydrous ethanol) of 1,450 ml per 10 kg of the raw sweet potato.

What is claimed is:

1. A process for saccharification of rootstocks and subsequent alcohol fermentation, which process comprises:

- a step of immersing rootstocks in a dilute acid solution to sterilize them;
- a step of crushing the resulting sterilized rootstocks;
- a step of macerating the crushed rootstocks through the action of macerating enzymes composed mainly of a polygalacturonase;
- a step of forming a slurry comprising liquid and starch derived solely from the product resulting from the action of said macerating enzymes on the crushed rootstocks;
- a step of adding a bacterial  $\alpha$ -amylase to the resulting slurry to dextrinize the starch contained therein; and
- a step of adding to the resulting dextrinized starch, glucoamylase as a saccharifying enzyme, and yeast for alcohol fermentation to effect saccharification and alcohol fermentation.

2. A process according to claim 1 wherein said rootstocks are at least one selected from the group consisting of white potatoes, sweet potatoes and cassava roots.

3. A process according to claim 1 wherein said polygalacturonase is pectin endopolygalacturonase.

4. A process according to claim 3 wherein said maceration enzymes contain small amounts of carboxyme-

thylcellulase, arabinoxylanase and arabinogalactanase, in addition to said pectin endopolygalacturonase.

5. A process according to claim 1 wherein said dextrinizing step by a bacterial  $\alpha$ -amylase is carried out at temperature of 80° C. to 90° C. and at pH of 5 or higher.

6. A process according to claim 1 wherein said maceration step by said maceration enzymes is carried out at temperature of 20° C. to 45° C. and at pH of 3.5 to 5.0.

7. A process according to claim 1 wherein said dilute acid is an aqueous solution of at least one compound selected from the group consisting of hydrochloric acid, sulfuric acid, and nitric acid.

8. A process according to claim 7, wherein the concentration of the acid is in the range of 0.02 to 0.08 normality.

9. A process according to claim 1 wherein a portion of the liquid in the slurry is removed before the dextrinizing step.

10. A process according to claim 9, wherein said rootstocks as raw material are white potatoes.

11. A process according to claim 1, wherein said dilute acid is an aqueous solution of acetic acid.

12. A process according to claim 11, wherein the concentration of the acid is in the range of 0.04 to 0.10 normality.

13. A process according to claim 1, wherein the rootstock is at least one of white potatoes and cassava root and the maceration enzymes are added in the ratio of 5 to 200 units per 100 g. of rootstocks.

14. A process according to claim 1, wherein the rootstock is sweet potatoes and the maceration enzymes are added in the ratio of 50 to 1,000 units per 100 g. of rootstocks.

15. A process according to claim 1, wherein the maceration step is conducted for 0.5 to 1 hour and the dextrinization step is conducted for about 1 to 15 minutes.

16. A process according to claim 1, wherein 4 to 16 units per gram of starch of bacterial  $\alpha$ -amylase is added.

17. A process according to claim 1, wherein the saccharification and alcohol fermentation step is conducted at 25° C. and at a pH of 4.5.

18. A process according to claim 1, wherein 2 to 10 units per gram of starch of the saccharifying enzyme are added.

19. A process according to claim 1, wherein small amounts of ammonium sulfate, potassium dihydrogen phosphate, calcium chloride and magnesium sulfate are added as nutrients for the yeast.

20. A batch process for saccharification of rootstocks and subsequent alcohol fermentation, which process comprises:

- a step of immersing rootstocks in a dilute acid solution to sterilize them;
- a step of crushing the resulting sterilized rootstocks;
- a step of macerating the crushed rootstocks through the action of blended enzyme agents comprising cellulase, hemicellulase, pectin endopolygalacturonase, glucoamylase and acidically active  $\alpha$ -amylase;
- a step of forming a slurry comprising liquid and starch derived solely from the product resulting from the action of said blended enzymes on the crushed rootstocks;
- a step of adding yeast for alcohol fermentation to the slurry to effect alcohol fermentation.

21. A process according to claims 1 or 20, wherein the pH of the dilute acid solution is in the range of 1.6 to 2.8.

22. A process according to claims 1 or 20, wherein the rootstocks are immersed in the dilute acid solution for a period of approximately 4 to 6 hours.

23. A process according to claims 1 to 10, wherein the rootstocks are crushed into particles having a size in the range of 8 to 20 meshes.

24. A process according to claim 20 wherein said rootstocks are selected from the group consisting of sweet potatoes and casava roots.

25. A process according to claim 20 wherein said blended enzyme agents are composed mainly of macerating enzymes and starch saccharifying amylase.

26. A process according to claim 20 wherein said blended enzyme agents contain per kg of rootstocks, 300 units or more of carboxymethylcellulase as said cellulase, 100 units or more of arabinoxylanase as said hemicellulase, as 100 units or more of said arabinogalactanase, 400 units or more of said pectin endopolygalacturonase in terms of a pectin viscosity-lowering activity, 1,500 units or more of said glucoamylase in terms of a starch-saccharifying activity, and 20 units or more of said acidically active  $\alpha$ -amylase in terms of a starch-gelatinizing activity determined by the iodometric method.

27. A process according to claim 20 wherein said maceration and saccharification of root-stocks by the action of said blended enzyme agents are carried out at temperature of 20° to about 45° C. and at pH of 3.5 to 5.0.

28. A process according to claim 27, wherein the temperature is 20° to 35° C.

29. A process according to claim 20 wherein said alcohol fermentation step is carried out at the same time with said maceration and saccharification step.

30. A process according to claim 20 wherein said blended enzyme agents are enzymes produced by fungi.

31. A process according to claim 30, wherein said fungi are selected from the group consisting of genus *Aspergillus* and genus *Rhizopus*.

32. A process according to claim 31, wherein the fungus is *Aspergillus niger*.

33. A batch for saccharification of rootstocks and subsequent alcohol fermentation comprising:

(a) immersing the rootstocks in a dilute acid to sterilize them;

(b) crushing the sterilized rootstocks from (a);

(c) macerating the crushed rootstocks from (b) through the action of macerating enzymes;

(d) forming a slurry comprising liquid and starch derived solely from the product resulting from the action of said macerating enzymes on the crushed rootstocks;

(e) dextrinizing the starch from (d) through the action of dextrinizing enzymes;

(f) saccharifying and fermenting the dextrinized starch from (d) through the action of a saccharifying enzyme and an alcohol fermentation yeast to effect saccharification and alcohol fermentation.

34. A process for the saccharification of white potatoes and subsequent alcohol fermentation comprising:

(a) immersing the white potatoes in a dilute acid to sterilize them;

(b) crushing the white potatoes from (a);

(c) macerating the crushed white potatoes through the action of macerating enzymes composed mainly of a polygalacturonase;

(d) a step of forming a slurry comprising liquid and starch derived solely from the product resulting from the action of said macerating enzymes on the crushed rootstocks;

(e) removing a portion of the liquid produced in (c);

(f) dextrinizing the starch from (c) through the action of bacterial  $\alpha$ -amylase;

(g) saccharifying and fermenting the dextrinized starch from (f) through the action of glucoamylase and an alcohol fermentation yeast to effect saccharification and alcohol fermentation respectively.

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# United States Patent [19]

Yoshizumi et al.

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[54] PROCESS FOR PRODUCING ALCOHOL BY  
FERMENTATION WITHOUT COOKING

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## [57] ABSTRACT

Alcohol is produced in a noncooking system by mixing a ground starchy material with mashing liquor at the range that the weight ratio of mashing liquor to ground starchy material is from 1.8 to 3.4 to form a slurry, without cooking, adding to the slurry at least 3.5 units as a saccharifying power of the enzyme preparation derived from a microorganism source, favorably *Rhizopus* sp., as a saccharifying agent, further adding an alcoholic fermenting yeast, and fermenting the slurry. Examples of the starchy material are cereals such as maize, sorghum, wheat, barley, rye, rice, barnyard millet, German millet, and common millet, and starchy rootcrops such as sweet potato and cassava.

34 Claims, No Drawings

## PROCESS FOR PRODUCING ALCOHOL BY FERMENTATION WITHOUT COOKING

This application is a continuation of application Ser. No. 326,283, filed Dec. 1, 1981, abandoned.

### BACKGROUND OF THE INVENTION

#### 1. Field of the Invention

This invention relates to a process for producing alcohol by saccharification and fermentation of a starchy material without cooking.

#### 2. Description of the Prior Art

It is the conventional practice to cook a mash consisting of a slurry of a ground starchy material at high temperatures such as 150° C. [*Industrial Fermentation* Vol.1, ed by L. A. Underkofler and R. J. Hickey, Chemical Publishing Co., Inc., 1954, p.17., *Trans. Am. Inst. Chem. Eng.*, 40, 421 (1944), *Food Can.*, 29, 23 (1969)] The purpose of cooking is to rupture the structure of the raw material grits thereby eluting starch from the grits to avoid a viscosity increase owing to gelatinization after eluting, aid in the actions of liquefying and saccharifying enzymes, and further to kill microorganisms harmful to saccharification and fermentation. After cooking, however, the mash must be cooled to about 25° to 35° C. which is the fermenting temperature, and the energy required for cooling is great. Hence, the total energy consumption including the energy spent for cooking is enormous in the conventional high temperature cooking process.

In recent years, the aggravated supply of energy caused by the increased price of petroleum has prompted much research work for development of new energy sources. In particular, much interest has been aroused in ethanol which can be renewably produced from biomass resources grown under the action of solar energy because it is expected to be a petroleum fuel substitute.

Some of the present inventors previously developed an energy-saving process for producing alcohol which involves cooking at a temperature of 75° to 85° C. (U.S. Pat. No. 4,092,434 issued May 30, 1978, G.B. Pat. No. 1,503,760 issued Mar. 15, 1978).

It is known on the other hand the processes for Japanese sake brewing and alcoholic production without cooking. [1. *J. Ferm. Ass. Japan* 10 319 (1952), 2. *J. Ferm. Ass. Japan* 21 83 (1963), 3. *J. Ferment. Technol.* 58 237 (1980), 4. *J. Brew. Soc. Japan* 75 858 (1980), 5. Abstract of Papers, Annual Meeting of the Society of Fermentation Technology of Japan, Osaka, November 1980 P4]. However, these techniques require some special operations such as acidification of mash (pH 3.5), which prevents contamination of harmful microorganisms (above-cited 1, 2, 3, 4). And the prior techniques require a longer period of the time for the saccharification and fermentation (above-cited 1, 2, 4, 5) or such prior techniques require complex process steps such as dialysis of a fermented broth (above-cited 3, 4) and are difficult to be accepted industrially.

### SUMMARY OF THE INVENTION

The present inventors have further investigated the process of the above-cited U.S. Pat. No. 4,092,434, G.B. Pat. No. 1,503,760 and now arrived at a novel energy-saving process for producing alcohol from starchy materials which requires no cooking steps.

According to this invention, there is provided a process for producing alcohol, which comprises mixing a ground starchy material with mashing liquor, at the range that the weight ratio of mashing liquor to ground starchy material is from 1.8 to 3.4 to form a slurry, without cooking the slurry, without adjusting the pH of the slurry, adding to the slurry a saccharifying enzyme preparation derived from a microorganism source, or malt, or both as a saccharifying agent in an amount of at least 3.5 units of saccharifying titer per one gram of raw material on wet basis (the saccharifying activity determined in accordance with JIS K-7001) on using the enzyme or the mixture of the enzyme and malt or at least 10 units as saccharifying titer on using malt only in terms of an enzyme titer and further mixing an alcoholic fermenting yeast; and then fermenting the slurry.

### DETAILED DESCRIPTION OF THE INVENTION

In the conventional industrial process, the weight ratio of the raw material vs mashing liquor is from 1:4.3 to 1:2.8, but in the process of this invention, the concentration of the raw material in the slurry is relatively high. Furthermore, it is favorable that in the initial stage, fermentation is carried out by maintaining the yeast concentration about  $2 \times 10^7$  cells/ml mash. In the conventional process, the final concentration of the resulting alcohol in the mash is about 11%, whereas it is about 15% in the process of this invention.

The process of the present invention is based on the surprising discovery that by adopting a relatively high mash concentration, a high fermentation yield and about 40% higher final alcohol concentration in the mash than in the conventional process can be obtained without cooking and any special pH adjustment.

The term "without cooking" or "noncooking", as used herein, means that no heat-treatment is carried out which will result in an increase in the viscosity of the slurry of the starchy material. In other words, this means that the raw material slurry is not treated at a temperature at which starch is converted to  $\alpha$ -starch or gelatinized. The temperature at which the viscosity increase begins differs depending upon the raw material and its concentration. Table 1 summarizes the relation between the initial concentration of a raw material and the starting temperature to increase the viscosity examined with regard to ground whole kernels of maize and corn starch by means of an amylograph of HAAKE INC. Thus, for example, when the weight ratio of the ground whole kernels of maize vs mashing water is 1:1.8, the slurry should be prepared without increasing viscosity due to gelatinization at a temperature lower than 55.8° C., and when the weight ratio of corn starch vs mashing water is 1:3.4, the slurry should be prepared at a temperature of less than 66.5° C.

TABLE 1

	1:3.4	1:2.9	1:2.4	1:2.1	1:1.8
Ground maize: water (by weight)					
Temperature of viscosity increase	66.5° C.	61.5° C.	60.0° C.	57.5° C.	55.8° C.
Corn starch: water (by weight)					
Temperature	66.5° C.	64.5° C.	63.0° C.	61.0° C.	62.0° C.

TABLE 1-continued

ture of  
viscosity  
increase

In a noncooking system, contamination by noxious microorganisms is likely to reduce the yield of fermentation or to stop the fermentation. However, according to the present invention, the concentration of fermentable sugars in the mash is enough but not excessive to the yeast fermentation. It seems that such the low concentration of sugars in the mash does not affect the saccharifying reaction by feedback inhibition.

Consequently, the process of the invention does not provide conditions suitable for the growth of noxious microorganisms such as spoilage bacteria, because of the shortage of assimilable sugars as a carbon source for the growth of bacteria, and there is scarcely any likelihood of contamination by the noxious microorganisms.

Furthermore, the process of the invention can save on energy for cooking, and is advantageous in regard to energy balance over conventional processes directed to the production of fuel alcohols from starchy materials.

Another advantage is that since the solids in the mash after fermentation have not experienced a heat history, they are easy to filter off, and can be separated by a simple filtration or decantation operation.

The process of this invention is described below in detail.

#### PREPARATION OF THE STARTING RAW MATERIAL

Examples of the starchy raw material used in this invention include cereals such as maize, sorghum, barley, wheat, rice, barnyard millet, German millet and common millet starchy rootcrops such as sweet potato and cassava, and raw starches separated from these materials.

The starting raw material may be a mixture of such materials. The cereals are used as ground whole kernels, or ground products of whole kernels from which the germ portion has been removed. The starchy rootcrops are used as ground products obtained from either raw starchy materials or from dried starchy materials. The particle diameter of the raw material should better be as fine as possible. Usually, it is sufficient that at least 30% of the starchy material has a particle size of not more than 840  $\mu$ m.

#### PREPARATION OF THE SLURRY

The ground raw material is mixed with mashing liquor as a weight ratio (ground raw material:mashing liquor) on wet basis of from 1:3.4 to 1:1.8 to form a slurry. Mashing liquor may be water or a mixture of water and a distillation residue of the mash which is known as "stillage" or stillage only.

If the concentration of the raw material in the slurry is lower than the above-specified limit (1:3.4), fermentation does not smoothly proceed. If it is higher than the above-specified limit (1:1.8), both the efficiency of saccharification and the yield of fermentation decrease. It is essential that the concentration of the raw material in the slurry should be within the above-specified range.

Use of stillage resulting from distilling off of alcohol from the mash after fermentation, in combination with water, is preferred because sugars, nitrogen, phosphorus, and other nutrients remaining in the stillage can be

utilized, and the use of stillage increases the buffering ability.

The stillage may be the one obtained by distilling off alcohol from the fermented mash by ordinary distillation under heat, or the one obtained by further removing crude solids from the above-mentioned type of stillage. The latter type is preferred, however.

Use of stillage together with water has been practiced heretofore, but the amount of stillage in the prior art is only up to 30% of the total amount of mashing liquor.

It is known that the fermentation residue left after distilling off alcohol by vacuum distilling is directly used as a raw material in the next cycle of alcoholic fermentation (Japanese Laid-Open Patent Publication No. 15691/1981). This method, however, requires a special operation and is not so easy to operate.

Since the process of this invention includes no cooking system, the viscosity of the slurry is low. Hence, the use of stillage in an increased proportion does not reduce the efficiency of transportation of the mash. Because of these advantages, the stillage can be used in a proportion of 50 to 100% instead of mashing water in the process of this invention.

The use of such a large amount of stillage produces an effect of facilitating the activation of yeast, particularly in the early stage of fermentation, promoting vigorous fermentation, and bring about good fermentation results.

The saccharifying agent to be added to the slurry is a saccharifying enzyme preparation derived from a microorganism source, or malt, or both. The amount of the saccharifying enzyme preparation is at least 3.5 units/g of the raw material on wet basis in terms of an enzyme titer. When malt is used, its amount is included in the amount of the raw material. Moreover, when malt is used as the sole saccharifying agent, the amount of malt is necessary to be equivalent at least 10 units/g as titer.

Existing and commercially available enzyme preparations can be used as the saccharifying enzyme preparations derived from microorganism source. They may be either the ones present in microorganism culture broths, or the ones extracted from the culture broths. For example, saccharifying agents derived from microorganism of the genus *Aspergillus* and *Rhizopus* are known to decompose raw starch, and are useful as the saccharifying agent in this invention. Particularly, enzyme preparations derived from *Rhizopus* sp. are suitable for the practice of the present invention because the pH of the slurry in this invention is 4.0 to 5.0 with an average of 4.6, and they have higher activity on saccharification of raw starch than those derived from *Aspergillus* sp. and exhibit strong saccharifying power.

The saccharifying activity of the enzyme preparations derived from microorganisms is expressed in terms of an enzyme titer measured in accordance with the method of JIS K-7001.

On the other hand, one unit of saccharifying power of malt is defined as the activity equivalent to produce 10 mg of maltose for 10 minutes at 40° C. from 1% soluble starch (pH 4.5).

In the present invention, the pH of the mash during fermentation is usually 4.0 to 5.0 with an average of about 4.5. On the other hand, the optimal pH for raw starch decomposition by malt is about 4.6. Accordingly, enzymes in the malt which participate in the decomposition of raw starch act in the vicinity of the optimal pH of malt.

### SACCHARIFICATION AND FERMENTATION

Usual alcohol fermenting yeasts can fully maintain their activities at a mash pH of 4.0 to 5.0. Accordingly, in the process of this invention, saccharification of starch is not retarded and alcoholic fermentation proceeds rapidly in spite of the fact that the starch is not converted to  $\alpha$ -starch nor gelatinized. This fact is one of the important points which the present inventors discovered.

tion is simple and economic method and the final concentration of the resulting alcohol in the mash is high such as 15% or over. Consequently, pure alcohol can be economically produced by conventional distillations.

The following examples illustrate the present invention more specifically.

### SACCHARIFYING ENZYME PREPARATIONS

Tables 2 shows the analysis of various saccharifying enzyme preparations from microorganisms.

TABLE 2

	Preparation code				
	A	B	C	D	E
	Origin of enzyme				
	Rhiz. sp. <sup>a</sup> (No. 212) +				
	Rhiz. sp. <sup>a</sup> (No. 204)	Rhiz. sp. <sup>a</sup> (No. 202)	Asp. sp. <sup>a</sup> (No. 107)	Asp. sp. <sup>a</sup> (No. 186)	Asp. sp. <sup>a</sup> (No. 171)
Liquefying power	105 U/g <sup>***</sup>	1850	103 U/g <sup>***</sup>	45 U/g <sup>***</sup>	118 U/g <sup>***</sup>
Dextrinizing power	400	5285	284	5347	518
Saccharifying power	2503	2236	1764	745	3763
Protease					
(acid)	985	3761	535	1236	13494
(neutral)	269	1262	1074	N.D. <sup>***</sup>	1969
(alkaline)	trace	1080	trace	N.D. <sup>***</sup>	1050
Cellulase	8.9	563	2.6	3.2	3.2
Pectinase	62	93	48	21	64

<sup>a</sup>Rhiz. sp.: *Rhizopus* sp.  
Asp. sp.: *Aspergillus* sp.  
<sup>\*\*\*</sup>U: units for Uolts  
<sup>\*\*\*</sup>N.D.: Not detected

The yeast starter used is a yeast cultivated in a conventional manner, and the amount of the yeast mixed to the slurry should be at least  $2 \times 10^6$  cells/ml of the mash as the initial yeast population. In an especially preferred embodiment, for a period of at least 10 hours from the starting time when the raw material slurry is combined with yeast starter, the yeast population is always maintained to at least  $2 \times 10^7$  cells/ml. of the mash. The method of achieving this is to add  $2 \times 10^7$  (cells/ml of mash) of yeast to the slurry, or to mix the yeast starter to a part of the slurry and after the yeast has grown, gradually add the remaining slurry. The latter method is preferred from the operational view point. By this operation, vigorous fermentation is carried out as soon as the raw material slurry is combined with yeast starter. Since alcoholic fermentation proceeds vigorously by the yeast, noxious microorganisms scarcely proliferate despite the absence of a cooking step, and contamination by spoilage microorganisms does not occur. The mash vigorously moves in a convectional manner. This convectional phenomenon causes uniformity of the density of the yeast and other components to provide preferred saccharifying and fermenting conditions. Specifically, eluting of starch from the ground raw materials is promoted. By these synergistic effects, fermentation yields equivalent to, or higher than, those in the conventional processes can be obtained within nearly the same periods of time as the conventional process by the noncooking process of the invention.

In some cases, for example depending upon the shape of the fermenter tank, the convection of the mash may be effected forcibly. If a raw material markedly contaminated by bacteria is used, the process of the invention may be carried out in the presence less than 320 ppm of sulfur dioxide. As mentioned above, the present inven-

Enzyme preparation (1 g) was added to distilled water (1000 ml) and then occasionally stirred for 1 hour at 30° C. The supernatant obtained by centrifugation (3000 r.p.m., 10 min.) was used as enzyme solution. Liquefying power, dextrinizing power, saccharifying power, protease, cellulase and pectinase were assayed by the methods of Wohlgemuth modified, Tufjisaka, JIS K 7001, Kunitz, Miller, and Willstatter-Schudel, respectively.

Table 3 shows the analysis of malts.

TABLE 3

Malt No.	A	B	C
Moisture (%)	4.5	4.2	4.6
Extract			
fine (%)	76.6	80.1	77.1
Coarse (%)	73.4	79.4	76.4
pH of wort	5.9	5.7	5.9
Saccharifying power (U/g)	46	110	113

Ground malt (1 g) was added to distilled water (1000 ml) and then occasionally stirred for 1 hour at 30° C. The supernatant obtained by centrifugation (3000 r.p.m., 10 min.) was used as enzyme solution. Saccharifying power was assayed by the method of JIS K 7001. One unit of saccharifying enzyme (amylase) is defined as the activity equivalent to produce 10 mg of maltose for 10 min. at 40° C. from 1% soluble starch (pH 4.5).

### PREPARATION OF SAMPLES

Starchy raw materials were ground in a dry condition and used for analysis. The degrees of grinding were as follows: The sweet potato and cassava used as raw materials were dried materials.

TABLE 4

Raw material	Particle size(*)	Degree of grinding						
		A (%)	B (%)	C (%)	D (%)	E (%)	F (%)	G (%)
Maize	above 840 $\mu$ m	69.0	28.8	21.6	20.8	10.2	4.4	0
	420-840 $\mu$ m	16.5	35.3	38.0	37.1	35.5	36.7	14.5
	250-420 $\mu$ m	3.8	10.8	10.9	10.9	12.6	12.3	11.9
	177-250 $\mu$ m	2.2	10.7	8.1	5.5	4.7	5.9	5.7
	149-177 $\mu$ m	1.9	3.6	4.5	1.2	0.3	1.1	1.3
	below 149 $\mu$ m	4.6	10.8	16.9	24.5	36.7	40.5	66.6
	Above 840 $\mu$ m	69.2	29.0	21.5	20.2	11.0	4.0	0
	420-840 $\mu$ m	16.3	34.8	37.6	37.5	35.3	36.5	14.2
Sorghum	250-420 $\mu$ m	6.0	11.0	10.8	11.2	12.0	11.8	11.5
	177-250 $\mu$ m	2.5	11.0	8.5	6.5	6.3	3.0	6.1
	149-177 $\mu$ m	2.0	3.8	4.7	1.8	1.0	1.2	1.7
	below 149 $\mu$ m	4.4	10.9	16.4	23.4	35.0	41.5	67.6
	above 840 $\mu$ m	67.5	30.5	20.0	19.1	9.1	3.7	0
	420-840 $\mu$ m	16.0	36.5	39.1	36.0	34.8	32.0	17.5
	250-420 $\mu$ m	6.0	9.0	11.0	12.5	13.7	13.5	12.0
	177-250 $\mu$ m	2.1	10.5	9.0	5.9	5.7	5.0	5.0
Wheat	149-177 $\mu$ m	3.0	3.5	5.0	1.5	1.2	1.8	1.6
	below 149 $\mu$ m	5.0	9.5	16.4	24.4	34.9	42.0	62.8
	above 840 $\mu$ m	68.5				9.8		
	420-840 $\mu$ m	14.8				35.0		
	250-420 $\mu$ m	6.5				13.0		
	177-250 $\mu$ m	1.9				5.2		
	149-177 $\mu$ m	2.5				1.3		
	below 149 $\mu$ m	5.8				35.7		
Rye	above 840 $\mu$ m	69.5				7.8		
	420-840 $\mu$ m	16.3				34.2		
	250-420 $\mu$ m	5.5				13.5		
	177-250 $\mu$ m	2.1				5.2		
	149-177 $\mu$ m	2.0				1.1		
	below 149 $\mu$ m	4.6				38.2		
	above 840 $\mu$ m	69.5		20.5		10.0		
	420-840 $\mu$ m	16.5		38.4		36.4		
Rice	250-420 $\mu$ m	5.2		12.1		12.3		
	177-250 $\mu$ m	2.0		6.5		4.0		
	149-177 $\mu$ m	2.0		4.1		1.1		
	below 149 $\mu$ m	4.8		18.1		36.2		
	above 840 $\mu$ m		32.5			11.0		
	420-840 $\mu$ m		37.0			34.4		
	250-420 $\mu$ m		13.2			12.0		
	177-250 $\mu$ m		6.7			5.0		
Common millet	149-177 $\mu$ m		2.0			1.0		
	below 149 $\mu$ m		8.6			36.6		
	above 840 $\mu$ m		30.5			10.0		
	420-840 $\mu$ m		36.1			36.0		
	250-420 $\mu$ m		9.8			13.0		
	177-250 $\mu$ m		10.0			4.0		
	149-177 $\mu$ m		4.0			1.8		
	below 149 $\mu$ m		9.6			35.2		
Common millet	above 840 $\mu$ m		31.5			9.8		
	420-840 $\mu$ m		38.0			37.5		
	250-420 $\mu$ m		12.5			11.8		
	177-250 $\mu$ m		7.1			5.7		
	149-177 $\mu$ m		3.0			1.8		
	below 149 $\mu$ m		7.9			33.4		
	above 840 $\mu$ m		38.4		19.4	10.6		
	420-840 $\mu$ m		32.5		42.9	39.8		
Cassava	250-420 $\mu$ m		12.1		17.9	23.6		
	177-250 $\mu$ m		8.2		10.6	13.6		
	149-177 $\mu$ m		3.2		2.7	4.8		

TABLE 4-continued

Raw material	Particle size(*)	Degree of grinding						
		A (%)	B (%)	C (%)	D (%)	E (%)	F (%)	G (%)
Sweet potato (dried chips)	below 149 $\mu$ m		5.6		6.5	7.6		
	above 840 $\mu$ m		32.5			9.0		
	420-840 $\mu$ m		34.0			32.5		
	250-420 $\mu$ m		13.0			10.3		
10	177-250 $\mu$ m		10.2			3.8		
	149-177 $\mu$ m		1.5			1.8		
	below 149 $\mu$ m		8.8			42.7		

(\*)Classified by using a standard mesh screen in accordance with JIS Z 8801-1964.

## EXAMPLE 1

185 g of ground product of each of the various cereals indicated in Table 5, 370 ml of water, 1.15 g (15.6 U/g of raw material as saccharifying power) of a saccharifying enzyme preparation A shown in Table 2 derived from *Rhizopus* sp. and 45 ml of yeast starter (*Saccharomyces* sp.;  $1.2 \times 10^8$  cells/ml.) were mixed with stirring in 1 liter of Meyer flask, and fermentation was carried out at 25° C. for 120 hours. The results of fermentation are shown in Table 5.

TABLE 5

30	Raw material	Degree of grinding (see Table 4)	Process of the invention			Conventional process <sup>(d)</sup> FE (%)	
			pH	TA <sup>(a)</sup> (ml)	Alc <sup>(b)</sup> (%)		FE <sup>(c)</sup> (%)
	Yellow dent maize	A	4.8	3.1	14.5	88.3	87.2
35	Sorghum	A	4.7	3.5	14.5	84.1	87.3
	Barley	A	4.8	3.5	13.7	88.0	86.8
	Wheat	A	4.8	3.6	13.7	87.5	87.0
	Rye	A	4.6	4.0	13.8	88.7	87.3
	Rice	A	4.9	3.0	16.2	91.2	90.8
40	German millet	B	4.8	3.2	12.5	85.0	84.3
	Barbadard millet	B	4.6	4.1	10.3	82.9	82.0
	Common millet	B	4.9	3.0	12.1	85.9	84.2

<sup>(a)</sup>TA: total acidity, ml of N/10 NaOH required for neutralizing 10 ml of mash.<sup>(b)</sup>Alc: alcohol content by volume<sup>(c)</sup>FE: fermentation efficiency =  $\frac{\text{Alcohol Produced (ml)}}{\text{Theoretical Alcohol (ml)}} \times 100(\%)$ <sup>(d)</sup>Conventional method: a conventional method in which the raw material was first cooked at a high temperature (150° C.) and thereafter saccharified and fermented.

## EXAMPLE 2

140 g of a ground product of a germ-removed fraction of each of the cereals shown in Table 6, 0.28 g (5.0 U/g of raw material) of the saccharifying enzyme preparation A derived from *Rhizopus* sp. as shown in Table 2, 80 ml of water, 322 ml of stillage and 25 ml of a yeast starter ( $1.1 \times 10^8$  cells/ml) were mixed with stirring, and fermentation was performed at 30° C. for 90 hours. The stillage used was obtained by subjecting the waste liquor discharged from the alcohol recovery tower to centrifugal separation to remove coarse solid components. The results are shown in Table 6.

TABLE 6

Raw material	Degree of grinding (see Table 4)	Process of the invention			Conventional process
		pH	TA (mM)	Alc (%)	FE (%)
Yellow dent maize	E	4.8	3.8	11.8	90.0
Sorghum	E	4.7	3.7	11.8	89.5

TABLE 6-continued

Raw material	Degree of grinding (see Table 4)	Process of the invention			Conventional process FE (%)
		pH	TA (ml)	Alc (%)	
Barley	E	4.7	3.7	11.6	87.7
Wheat	E	4.6	3.9	11.5	86.3
Rye	E	4.6	4.0	11.6	86.9
Rice	E	4.9	3.5	13.6	92.5

## EXAMPLE 3

185 g of a whole grain product of white dent maize and Sorghum, 0.33 g (4 U/g of raw material) of the saccharifying enzyme B shown in Table 2 derived from *Rhizopus* sp., 80 mg of  $K_2S_2O_8$ , 370 ml of water and 25 ml of yeast starter ( $1.2 \times 10^8$  cells/ml) were mixed with stirring, and fermentation was performed at 32° C. for 110 hours. The results are shown in Table 7.

TABLE 7

Raw material	Degree of grinding (see Table 4)	Process of the invention			Conventional process FE (%)
		pH	TA (ml)	Alc (%)	
White dent maize	E	4.9	3.0	14.5	87.1
Sorghum	E	4.9	3.0	14.5	87.2

## EXAMPLE 4

Four liters of yeast starter ( $1.2 \times 10^8$  cell/ml) was put into a 100-liter fermentation tank. A whole grain ground product (the degree of grinding F) of sorghum and mashing liquor were mixed in a weight ratio of 1:2, and a saccharifying enzyme preparation D shown in Table 2 derived from *Asp. sp.* in an amount of 20 units per gram of sorghum as a saccharifying power was added to form a slurry. The slurry was gradually added so that the number of yeast cells in the mash after the addition was kept always at more than  $2 \times 10^7$  cells/ml. The total amount of the mash was adjusted to 84 liters, and fermentation was carried out at 32° C. for 96 hours. The mashing liquor used in this experiment was a mixture of water and stillage in a ratio of 7:3 by volume. The stillage used was the one obtained by performing the same treatment as described in Example 2. The results are given in Table 8 which shows changes with time in the amount of direct reducing sugar in the mash, and in Table 9 which shows changes with time in the number of yeast cells in the mash and the results of fermentation.

TABLE 8

Time (hours) from the start of addition of the raw material	Process of the invention	Conventional process
1	1.10%	6.79%
10	1.63%	6.45%
24	0.17%	2.52%
48	0.14%	0.18%
72	0.12%	0.12%
96	0.09%	0.10%

TABLE 9

5	Process of the invention										Conventional process
	Number of yeast cells in the mash ( $\times 10^7$ cells/ml) Time (hours) from the start of addition of the raw material						Results of fermentation			FE (%)	
	1	5	10	24	48	72	96	pH	TA (ml)	Alc (%)	
4.2	2.0	6.8	18	13	10	8	5.0	3.0	15.0	89.5	88.2

## EXAMPLE 5

400 ml of yeast starter ( $1.2 \times 10^8$  cells/ml) was put into a 10-liter glass fermentation tank (inside diameter 20 cm, height 40 cm). Each of the various materials in the ground state shown in Table 10 and mashing liquor were mixed in a weight ratio of 1:2, and 23 units, as a saccharifying power, per gram of the raw material of the saccharifying enzyme preparation D shown in Table 2 derived from *Asp. sp.* was added to prepare a slurry. The slurry was gradually added so that the number of yeast cells in the mash after the addition was always kept at more than  $2 \times 10^7$  cells/ml. The total amount of the mash was adjusted to 8.4 liters, and fermentation was carried out at 35° C. for 110 hours. The mashing liquor used in this experiment was a mixture of water and stillage in a ratio of 1:1, and the stillage was the one obtained by performing the same treatment as in Example 2.

The results are shown in Table 10.

TABLE 10

Raw material	Degree of grinding (see Table 4)	Process of the invention										Conventional process FE (%)
		Number of yeast cells ( $\times 10^7$ cells/ml in the mash)					Results of fermentation					
		Time (hours) from the start of addition of the raw material					pH	TA (ml)	Alc (%)	FE (%)		
		1	5	10	24	pH						
											TA (ml)	
Maize	B	4.0	2.2	6.0	18	4.9	3.8	14.4	88.1	87.6		
Sorghum	B	4.5	2.0	5.9	18	4.7	4.0	14.4	87.8	87.5		
Barley	B	5.0	2.9	7.8	21	4.9	3.5	14.1	87.6	86.5		
Wheat	E	4.8	2.6	6.2	17	4.8	3.7	14.1	87.4	86.8		
Rye	E	4.7	2.4	7.0	18	4.6	4.1	14.2	88.0	87.1		
Rice	E	3.5	3.7	6.9	20	5.0	3.5	16.5	90.5	90.2		
German millet	E	6.0	4.2	8.0	18	4.7	3.9	12.9	84.7	83.6		
Buryard millet	E	5.0	2.8	6.1	17	4.7	4.5	10.6	82.5	81.5		
Common millet	E	4.5	2.1	6.1	19	4.9	3.9	12.4	86.0	84.9		

## EXAMPLE 6

400 ml of yeast starter ( $1.1 \times 10^8$  cells/ml) was put into a 10-liter glass fermentation tank (inside diameter 20 cm, height 40 cm). A ground product of each of the raw materials shown in Table 11 and mashing liquor were mixed in a weight ratio of 1:1.8, and 4 units, as a saccharifying power, per g of the raw material of the saccharifying enzyme preparation E shown in Table 2 derived from *Asp. sp.* was added to form a slurry. The slurry was gradually added so that the number of yeast cells in the mash after the addition was always kept at more than  $2 \times 10^7$  cells/ml. The total amount of the

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mash was adjusted to 8.4 liters, and fermentation was carried out at 32° C. for 120 hours. The mashing liquor used in this experiment was a mixture of water and stillage in a ratio of 8:2. The stillage was the one obtained by performing the same treatment as in Example 2.

The results are shown in Table 11.

TABLE 11

Raw material	Degree of grinding (see Table 4)	The process of the invention										Conventional process
		Number of yeast cells ( $\times 10^7$ cells/ml in the mash)					Results of fermentation					
		Time (hours) after the start of addition of the raw material										
		1	5	10	24	pH	TA (ml)	Alc (%)	FE (%)	FE (%)		
Maize	G	4.5	4.0	7.2	19	4.9	3.7	15.3	87.5	87.7		
Sorghum	G	4.7	3.1	6.0	17	4.8	3.2	15.6	87.6	87.3		
Rice	O	1.8	2.0	7.4	18	4.9	3.4	17.1	89.7	89.5		

## EXAMPLE 7

120 g of a starch fraction separated from each of the raw materials shown in Table 12, 1.34 g (25 U/g of raw material) of the saccharifying enzyme preparation B shown in Table 2 derived from *Rhizopus* sp., 408 ml of stillage, and 25 ml of yeast starter were mixed with stirring, and fermentation was carried out at 35° C. for 96 hours. The stillage used in this experiment was the liquid discharged from the alcohol recovery tower. The results are shown in Table 12.

TABLE 12

Raw material	Process of the invention				Conventional process
	pH	TA (mM)	Alc (%)	FE (%)	
Maize	4.5	4.0	12.3	90.0	87.7
Sorghum	4.4	4.1	12.3	89.5	88.0
Barley	4.4	4.2	12.2	88.6	87.9
Wheat	4.3	4.3	12.2	88.5	87.0
Rye	4.2	4.3	12.3	89.1	88.3
Rice	4.7	4.1	12.5	92.1	90.0
Sweet potato	4.3	4.0	12.3	89.2	86.8
Cassava	4.2	4.2	12.3	89.5	88.5

## EXAMPLE 8

1540 g of a whole grain ground product (the degree of grinding E) of yellow dent maize, 1260 g of commercial corn starch, 10.5 g (8.4 U/g of raw material) of saccharifying enzyme preparation derived from *Rhizopus* sp. (B in Table 2), 5.6 liters of water and 2.4 liters of stillage and 500 ml of yeast starter ( $1.0 \times 10^8$  cells/ml)

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were mixed, and fermentation was carried out for 100 hours at 32° C. The results are shown in Table 13.

TABLE 13

Raw material	Process of the invention				Conventional process
	pH	TA (mM)	Alc (%)	FE (%)	
Yellow dent maize and commercial corn starch	4.6	6.1	12.2	88.0	87.1

## EXAMPLE 9

126 g of a whole grain ground product (the degree of grinding B) of yellow dent maize, 14 g (4.6 U/g of raw material) of ground malt (A shown in Table 3), 0.22 g (3.5 U/g of raw material) of saccharifying enzyme preparation from *Rhizopus* sp. (B in Table 2), 80 mg of  $K_2S_2O_8$ , 402 ml of water and 25 ml of yeast starter ( $1.1 \times 10^8$  cells/ml) mixed with stirring, and fermentation was carried out at 30° C. for 96 hours. The results are shown in Table 14.

TABLE 14

Raw material	Process of the invention				Conventional process
	pH	TA (mM)	Alc (%)	FE (%)	
Yellow dent maize and malt	4.7	3.0	11.2	88.2	87.3

## EXAMPLE 10

670 ml of yeast starter ( $1.0 \times 10^8$  cells/ml) was put into a 10-liter glass fermentation tank (inside diameter 20 cm, height 40 cm). Ground maize, ground malt (B in Table 3; 22 U/g of raw material as saccharifying power), and mashing liquor were mixed in a weight ratio (maize:malt:mashing liquor) or 4:1:14.5, and 320 ppm of  $K_2S_2O_8$  was added to form a slurry. The slurry was gradually added so that the number of yeast cells in the mash after the addition was kept always at more than  $2 \times 10^7$  cells/ml. The total amount of the mash was adjusted to 8.7 liters, and fermentation was carried out at 32° C. for 96 hours.

The mashing liquor used in this experiment was a mixture of water and stillage in a ratio of 7:3. The results are shown in Table 15.

TABLE 15

Raw material	Degree of grinding (see Table 4)	Process of the invention											Conventional process	
		Number of the yeast cells in the mash ( $\times 10^7$ cells/ml)								Results of fermentation				FE (%)
		Time (hours) after the start of addition of the raw material								pH	TA (ml)	Alc (%)		
		1	5	10	24	48	72	96						
Maize and malt	B	4.0	2.4	6.0	19	15	7.2	5	4.8	3.0	10.5	87.3	86.8	

A similar experiment to the above was carried out using sorghum, barley, wheat, rye and rice. The results are shown in Table 16.

TABLE 16

Raw material	Degree of grinding (see Table 4)	Process of the invention								Conventional process
		Number of yeast cells in the mash ( $\times 10^7$ cells/ml)				Results of fermentation				
		Time (hours) from the start of addition of				pH	TA (ml)	Alc (%)	FE (%)	
		the raw material								
		1	5	10	24					
Sorghum and malt	B	3.8	2.0	5.8	18	5.0	3.2	10.6	87.0	87.0
Barley and malt	C	5.0	2.8	7.0	19	4.9	3.1	10.5	87.2	86.5
Wheat and malt	E	4.6	2.2	6.4	19	4.9	3.0	10.5	87.1	87.0
Rye and malt	E	4.5	2.1	6.5	17	4.9	3.3	10.6	88.0	87.5
Rice and malt	C	5.6	2.7	7.5	20	4.9	3.0	11.9	89.7	89.0

## EXAMPLE 11

800 ml of yeast starter ( $1.3 \times 10^8$  cells/ml) was put into a 10-liter glass fermentation tank (inside diameter 20 cm, height 40 cm).

A ground product of each of the raw materials shown in Table 17 ground malt (C in Table 3; 11.6 U/g of raw material was saccharifying power) and water were mixed in a weight ratio of 8.7:1:22.8 (a ground product:ground malt:water) and 160 ppm of  $K_2S_2O_5$  was further added to form a slurry.

The slurry was gradually added to the tank so that the number of yeast cells in the mash after the addition was kept always at more than  $2 \times 10^7$  cells/ml. The total amount of the mash was adjusted to 8.8 liters, and fermentation was carried out at 28° C. for 120 hours. The results are shown in Table 17.

TABLE 17

Raw material	Degree of grinding (see Table 4)	Process of the invention				Conventional process	
		Number of yeast cells in the mash ( $\times 10^7$ cells/ml)					
		pH	TA (tit)	Alc (%)	FE (%)		FE (%)
Maine and malt	G	4.6	3.3	11.7	86.5	86.6	
Sorghum and malt	G	4.5	4.0	11.9	86.8	86.5	
Rice and malt	G	4.8	4.1	12.9	87.5	87.3	

## EXAMPLE 12

185 g of ground cassava (dried), 370 ml of water, 200 ml of stillage, 0.58 g (7.8 U/g of raw material) of the saccharifying enzyme preparation derived from *Rhizopus* sp. (A in Table 2), 45 ml of yeast starter ( $1.2 \times 10^8$  cells/ml) were mixed with stirring, and fermentation was carried out at 30° C. for 96 hours. The results are shown in Table 18.

TABLE 18

Degree of grinding (see Table 4)	Process of the invention				Conventional process
	Number of yeast cells in the mash ( $\times 10^7$ cells/ml)				
	pH	TA (ml)	Alc (%)	FE (%)	
B	4.2	3.1	12.5	87.8	88.2
D	4.3	3.3	12.6	88.3	88.4
E	4.3	3.1	12.7	89.6	88.5

## EXAMPLE 13

185 g of ground sweet potato (dried chips), 305 ml of water, 165 ml of stillage, 0.32 g (3.9 U/g of raw material) of the saccharifying enzyme derived from *Rhizopus* sp. (B in Table 2), and 40 ml ( $1.0 \times 10^8$  cells/ml) of yeast starter were mixed with stirring, and fermentation was carried out at 32° C. for 120 hours. The results are shown in Table 19.

TABLE 19

Degree of grinding (see Table 4)	Process of the invention				Conventional process
		TA	Alc	FE	FE
	pH	(ml)	(%)	(%)	(%)
B	4.5	4.1	12.2	91.2	91.8
E	4.6	4.0	12.3	92.0	91.5

## EXAMPLE 14

112 g of a ground product of each of the raw materials (dried) shown in Table 20, 28 g of ground malt (B in Table 3; 22 U/g of raw material), 200 ml of water, 200 ml of stillage, 320 mg of  $K_2S_2O_5$ , and 25 ml ( $1.3 \times 10^8$  cells/ml) of yeast starter were mixed, and fermentation was carried out at 28° C. for 120 hours. The results are shown in Table 20.

TABLE 20

Raw material	Degree of grinding (see Table 4)	Process of the invention				Conventional process
		TA	Alc	FE	FE	
		pH	(ml)	(%)	(%)	
Cassava and malt	E	4.3	2.4	12.6	87.8	86.8
Sweet potato and malt	E	4.5	4.3	11.1	90.2	90.6

## EXAMPLE 15

400 ml of yeast starter ( $1.3 \times 10^8$  cells/ml) was put into a 10-liter glass fermentation tank (inside diameter 20 cm, height 40 cm).

Separately, a ground product of germ-removed fraction of each of the cereals shown in Table 21 and mashing liquor were mixed in a weight ratio of 1:2.5, and the enzyme preparation derived from a microorganism (C in Table 2; 5.9 U/g of raw material) was added to form a slurry. The slurry was then added gradually to the tank so that the number of yeast cells in the mash after the addition was kept always at more than  $2 \times 10^7$  cells/ml. The total amount of the mash was adjusted to 7.6 liters, and fermentation was carried out at 30° C. for 120 hours. The liquor used in this experiment was a mixture of water and stillage in a ratio of 1:1. The results are shown in Table 21.

TABLE 21

Raw material	Degree of grinding (see Table 4)	Process of the invention										Conventional process
		Number of yeast cells in the mash ( $\times 10^7$ cells/ml) Time (hours) after the start of addition of the raw material					Results of fermentation					
							pH	TA (ml)	Alc (%)	FE (%)	FE (%)	
		1	5	10	24							
Maize	G	6.8	4.2	8.3	19	4.8	3.6	12.8	88.0	87.0		
Sorghum	E	6.2	2.1	7.1	17	4.9	3.5	12.9	88.1	87.9		
Rice	G	7.1	4.5	7.8	20	5.0	3.2	14.7	92.7	92.2		

#### What is claimed is:

1. A process for producing a fermented mash containing from 12.2 to 17.1 V/V% of alcohol, which comprises mixing at least one non-heated raw cereal with a mashing liquor in a weight ratio (weight of said raw cereal: weight of mashing liquor) of from 1:3.4 to 1:1.8 to form a slurry; thereafter without heating the slurry, without adjusting the pH of the slurry and without adding liquefying enzyme, adding a saccharifying enzyme preparation derived from a microorganism source in an amount of at least 3.5 units of saccharifying titer per gram of said raw cereal on a wet basis to the slurry, adding an alcoholic fermenting yeast having an initial concentration of at least  $2 \times 10^7$  cells/ml of slurry, and fermenting the slurry at a pH of from 4.0 to 5.0, at a temperature of 25° to 35° C. for 90 to 120 hours.
2. The process of claim 1, wherein the raw cereal is selected from the group consisting of maize, sorghum, wheat, barley, rye, rice, barnyard millet, German millet and common millet.
3. The process of claim 1, wherein the mashing liquor is water, or a mixture of water and distillers stillage.
4. The process of claim 3, wherein the mashing liquor is completely substituted by the distillers stillage.
5. The process of claim 1, wherein the enzyme preparation is derived from a microorganism of the genus *Rhizopus*.
6. The process of claim 1, wherein the saccharifying titer of the enzyme preparation is 3.5 to 25 units.
7. The process of claim 1, further comprising adding less than 320 ppm of sulfur dioxide to the slurry.
8. The process according to claim 1, wherein at least 30% of the raw cereal has a particle size not exceeding 840  $\mu$ m.
9. A process for producing a fermented mash containing from 12.2 to 17.1 V/V% of alcohol, which comprises mixing at least one non-heated raw cereal and ground malt with a mashing liquor in a weight ratio (weight of said raw cereal including malt: weight of mashing liquor) of from 1:3.4 to 1:1.8 to form a slurry; thereafter without heating the slurry, without adjusting the pH of the slurry and without adding liquefying enzyme, adding a saccharifying enzyme preparation derived from a microorganism source wherein the total saccharifying power of the malt and enzyme preparation based on units of the saccharifying titer of the enzyme preparation is at least 3.5 units, adding an alcoholic fermenting yeast having an initial concentration of at least  $2 \times 10^7$  cells/ml of slurry, and fermenting the slurry at a pH of from 4.0 to 5.0, at a temperature of from 25° to 35° C. for 90 to 120 hours.
10. The process of claim 9, wherein the raw cereal is selected from the group consisting of maize, sorghum,

barley, wheat, rye, rice, barnyard millet, German millet and common millet.

11. The process of claim 9, wherein the mashing liquor is water, or a mixture of water and distillers stillage.

12. The process of claim 11, wherein the mashing liquor is completely substituted by the distillers stillage.

13. The process of claim 9, wherein the enzyme preparation is derived from a microorganism of the genus *Rhizopus*.

14. The process of claim 9, wherein the saccharifying titer of the enzyme preparation is 0.3 to 10 units.

15. The process of claim 9, further comprising adding less than 320 ppm of sulfur dioxide to the slurry.

16. The process according to claim 9, wherein at least 30% of the raw cereal has a particle size not exceeding 840  $\mu$ m.

17. A process for producing a fermented mash containing from 12.2 to 17.1 V/V% of alcohol, which comprises mixing at least one non-heated raw cereal and ground malt with a mashing liquor in a weight ratio (weight of said raw cereal including malt: weight of mashing liquor) of from 1:3.4 to 1:1.8 to form a slurry, the amount of malt being sufficient to provide at least 10 units of saccharifying power per gram of said raw cereal; thereafter without heating the slurry, without adjusting the pH of the slurry and without adding liquefying enzyme, adding an alcoholic fermenting yeast having an initial concentration of at least  $2 \times 10^7$  cells/ml of slurry, and fermenting the slurry at a pH of from 4.0 to 5.0, at a temperature of 25° to 35° C. for 90 to 120 hours.

18. The process of claim 17, wherein the raw cereal is selected from the group consisting of maize, sorghum, barley, rye, rice, barnyard millet, German millet and common millet.

19. The process of claim 17, wherein the mashing liquor is water, or a mixture of water and distillers stillage.

20. The process of claim 19, wherein the mashing liquor is completely substituted by the distillers stillage.

21. The process of claim 17, wherein the amount of malt is sufficient to provide from 10 to 25 units of saccharifying power per gram of starchy material.

22. The process of claim 17, further comprising adding less than 320 ppm of sulfur dioxide to the slurry.

23. The process according to claim 17, wherein at least 30% of the raw cereal has a particle size not exceeding 840  $\mu$ m.

24. A process for producing a fermented mash containing from 12.2 to 17.1 V/V% of alcohol, which comprises mixing at least one non-heated raw cereal with a mashing liquor in a weight ratio (weight of said raw cereal: weight of mashing liquor) of from 1:3.4 to 1:1.8 to form a slurry; thereafter without heating the slurry, without adjusting the pH of the slurry and without adding liquefying enzyme, adding a saccharifying enzyme preparation derived from a microorganism source having at least 3.5 units of saccharifying titer per gram of said raw cereal on a wet basis to the slurry, and mixing part of the slurry with yeast to provide a yeast population of at least  $2 \times 10^7$  cells/ml of the slurry and then adding gradually the remainder of the slurry to the yeast containing slurry so that the yeast population in the slurry is always in the amount of at least  $2 \times 10^7$  cells/ml, and fermenting the slurry at a pH of from 4.0 to 5.0, at a temperature of 25° to 35° C. for 90 to 120 hours.

25. The process according to claim 24, wherein at least 30% of the raw cereal has a particle size not exceeding 840  $\mu\text{m}$ .

26. A process for producing a fermented mash containing from 12.2 to 17.1 V/V% of alcohol, which comprises mixing at least one non-heated raw cereal and ground malt with a mashing liquor in a weight ratio (weight of said raw cereal including malt: weight of mashing liquor) of from 1:3.4 to 1:1.8 to form a slurry; thereafter without heating the slurry, without adjusting the pH of the slurry and without adding liquefying enzyme, adding a saccharifying enzyme preparation derived from a microorganism source wherein the total saccharifying power of the malt and enzyme preparation based on units of the saccharifying titer of the enzyme preparation is at least 3.5 units and mixing part of the slurry with yeast to provide a yeast population of at least  $2 \times 10^7$  cells/ml of the slurry and then adding gradually the remainder of the slurry to the yeast containing slurry so that the yeast population in the resulting slurry is always in the amount of at least  $2 \times 10^7$  cells/ml, and fermenting the slurry at a pH of from 4.0 to 5.0, at a temperature of 25° to 35° C. for 90 to 120 hours.

27. The process according to claim 26, wherein at least 30% of the raw cereal has a particle size not exceeding 840  $\mu\text{m}$ .

28. A process for producing a fermented mash containing from 12.2 to 17.1 V/V% of alcohol, which comprises mixing at least one non-heated raw cereal and ground malt with a mashing liquor in a weight ratio (weight of said raw cereal including malt: weight of mashing liquor) of from 1:3.4 to 1:1.8 to form a slurry, the amount of malt being sufficient to provide at least 10 units of saccharifying power per gram of said raw cereal; thereafter without heating the slurry, without adjusting the pH of the slurry and without adding liquefying enzyme, mixing part of the slurry with yeast to provide a yeast population of at least  $2 \times 10^7$  cells/ml of

the slurry and then adding gradually the remainder of the slurry to the yeast containing slurry so that the yeast population in the resulting slurry is always in the amount of at least  $2 \times 10^7$  cells/ml, and fermenting the slurry at a pH of from 4.0 to 5.0, at a temperature of 25° to 35° C. for 90 to 120 hours.

29. The process according to claim 28, wherein at least 30% of the raw cereal has a particle size not exceeding 840  $\mu\text{m}$ .

30. A process for producing a fermented mash containing from 12.2 to 17.1 V/V% of alcohol, which comprises mixing at least one non-heated ground raw cereal with a mashing liquor which contains 50 to 100% of distillers stillage in a weight ratio (weight of said raw cereal: weight of mashing liquor) of from 1:3.4 to 1:1.8 to form a slurry; thereafter without heating the slurry, without adjusting the pH of the slurry, and without adding liquefying enzyme, adding a saccharifying enzyme preparation derived from a microorganism source in an amount of at least 3.5 units of saccharifying titer per gram of said raw cereal on a wet basis to the slurry, adding an alcoholic fermenting yeast having an initial concentration of at least  $2 \times 10^7$  cells/ml of slurry and fermenting the slurry at a pH of from 4.0 to 5.0 at a temperature of 25° to 35° C. and from 90 to 120 hours.

31. The process of claim 30, wherein the raw cereal is selected from the group consisting of maize, sorghum, wheat, barley, rye, rice, barnyard millet, German millet and common millet.

32. The process of claim 30, wherein the enzyme preparation is derived from a microorganism of the genus *Rhizopus*.

33. The process of claim 30, wherein the saccharifying titer of the enzyme preparation is 3.5 to 25 units.

34. The process of claim 30, further comprising adding less than 320 ppm of sulfurous dioxide to the slurry.

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# United States Patent [19]

Lützen

[11] 4,316,956

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## [54] FERMENTATION PROCESS

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[73] Assignee: Novo Industri A/S, Denmark

[21] Appl. No.: 119,034

[22] Filed: Feb. 6, 1980

[51] Int. Cl.<sup>3</sup> ..... C12P 19/20

[52] U.S. Cl. .... 435/96; 435/99;  
435/161

[58] Field of Search ..... 435/96, 42, 99, 161,  
435/162, 813; 426/11, 13, 14, 29, 494

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*Primary Examiner*—R. B. Penland

*Attorney, Agent, or Firm*—Fidelman, Wolffe & Waldron

## [57] ABSTRACT

This invention relates to a novel fermentation process and in particular, to fermentative production of ethanol producing in the presence of non-gelled, or granular, starch particles, alpha-amylase and a glucoamylase; characteristic of the present process is recycle of enzymes for renewed use in fermentation, usually through termination of the fermentation prior to complete disappearance of the granular starch particles, and recovery for use anew of the unconsumed starch, along with enzymes thereon. Fermentation according to practice of this invention proceeds at a near to linear rate in the main.

8 Claims, 9 Drawing Figures

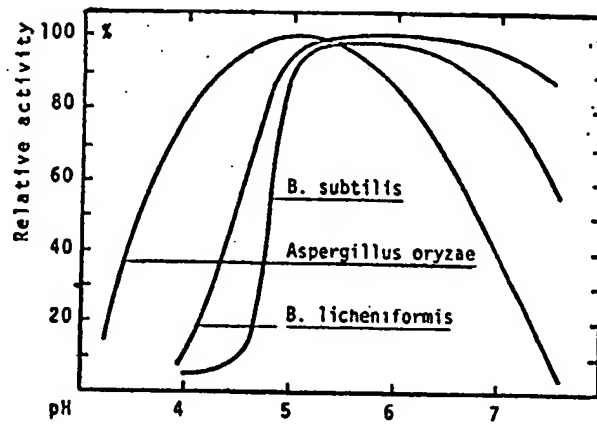


FIG. 1

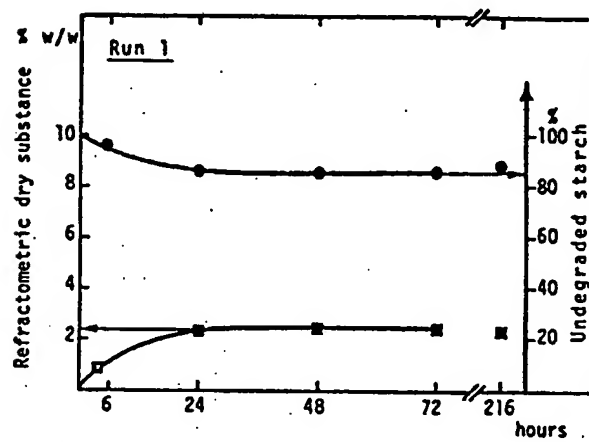


FIG. 2

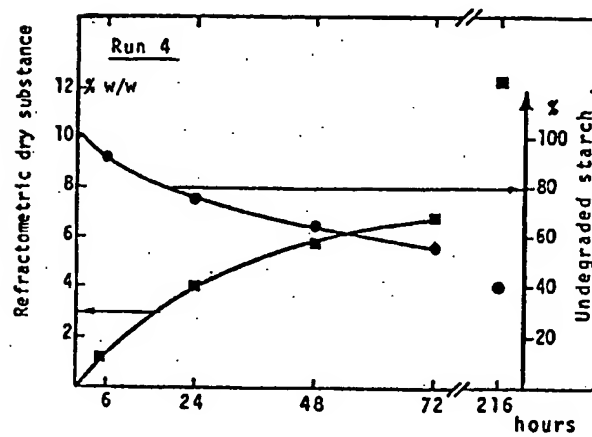


FIG. 3

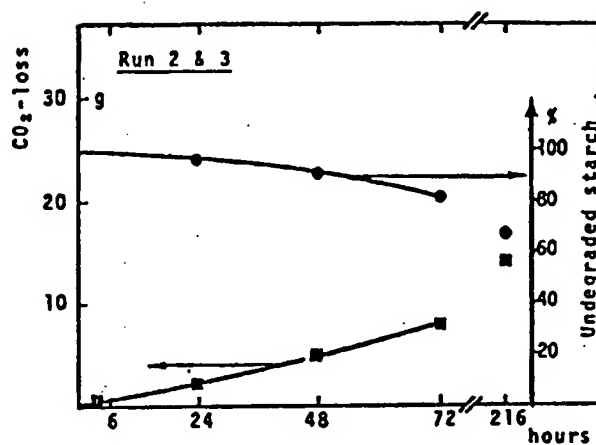


FIG. 4

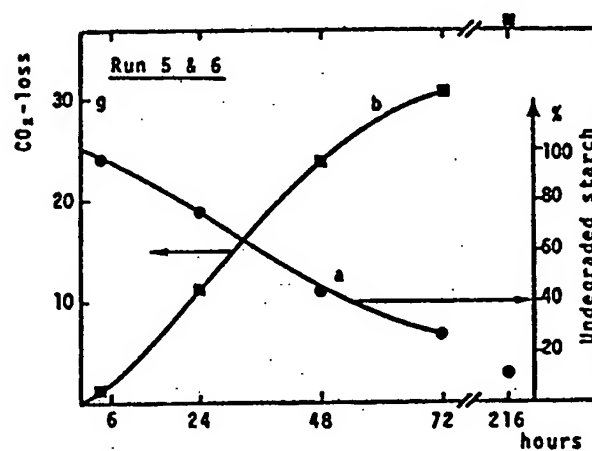


FIG. 5

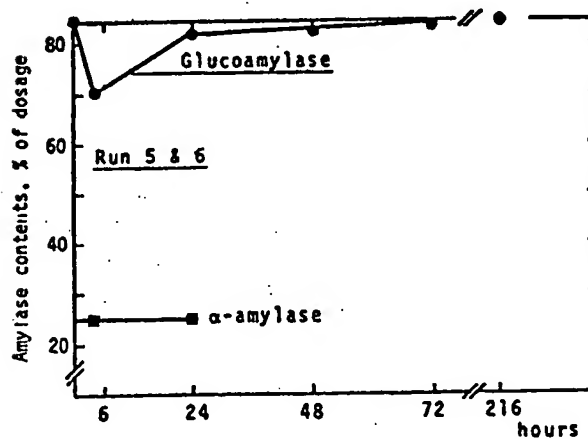


FIG. 6

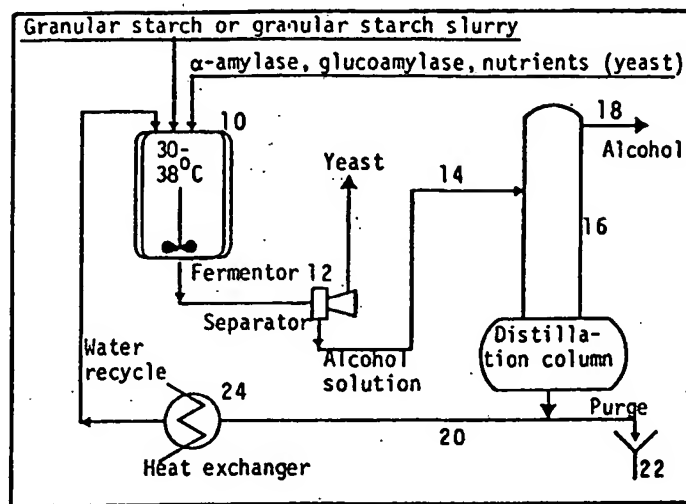


FIG. 7

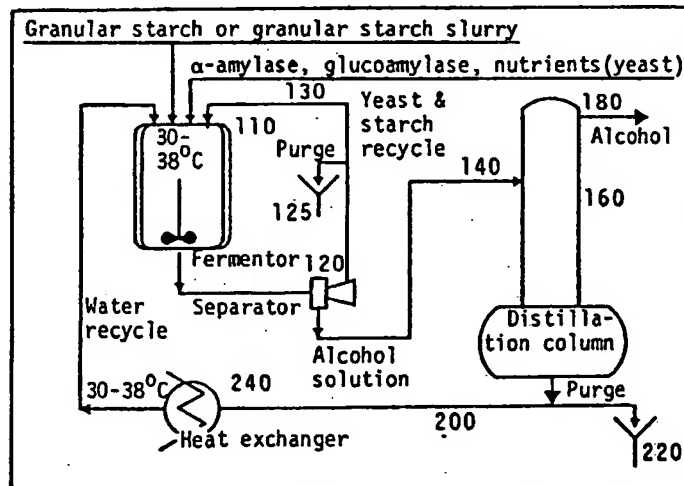


FIG. 8

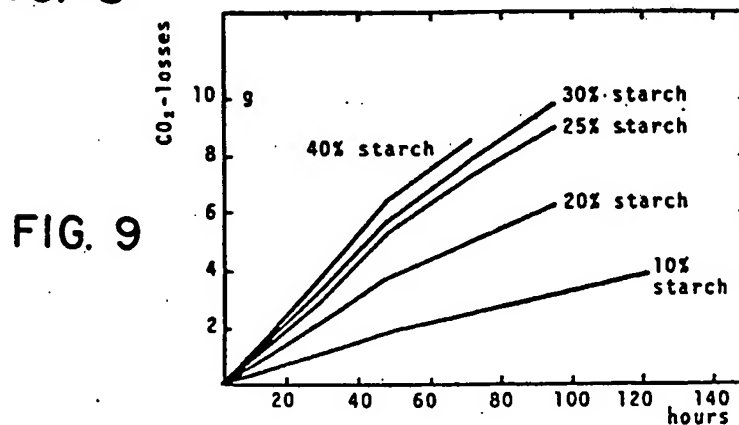


FIG. 9

## FERMENTATION PROCESS

## BACKGROUND OF THE INVENTION

The conventional fermentation for the production of an ethanol containing solution must operate within the conditions required for cultivating the ethanol producing microorganism, e.g., *Saccharomyces cerevisiae* including, for example, maintenance of pH between about 3 and 7, maintenance of a temperature range between about 25° C. and 38° C., commencement of the fermentation with a wort containing not more than about 20% glucose by weight and avoidance of any efforts to generate more than about 10% by weight of alcohol in the fermentation broth.

In the great many instances wherein starch or a starchy substance such as corn grits is the source of the glucose consumed in the fermentation, the process includes a starch liquefaction and hydrolysis sequence to convert the (solid) starch into a glucose solution that becomes, in part at least, the growth medium for the yeast.

While starch conversion into glucose and maltose for fermentation purposes has been carried out on a large scale for eons in commercial practice, the procedures employed in prior art practices rarely convert starch completely into fermentable sugars. Even relatively minor deficiencies in conversion of starch into the fermentables, maltose and glucose, have adverse affect on the fermentation, the least of which is lower yield than would otherwise result, and/or a greater processing expense. For example, the traditional process for making beer wherein grains are hydrolyzed by malt results in a wort with a significant nonfermentable polysaccharide content, and, in turn, a beer with a significant polysaccharide content.

This situation has, of course, received considerable attention from workers in the pertinent arts.

On the whole, it is fair to state that starch liquefaction and hydrolysis procedures capable of producing a pure glucose syrup are available, as for example, liquefaction of starch according to the procedures described in U.S. Pat. No. 3,912,590 followed by saccharification according to the procedures described in U.S. Pat. No. 4,017,363. However, all of the starch liquefaction and hydrolysis procedures known to the inventor hereof can be criticized for requiring moderate to large quantities of thermal energy.

It should be noted moreover that fermentation of starch derived glucose syrups, whether of high purity or otherwise, face the microorganism with the presence of a very large excess of the glucose nutrient at the onset of the fermentation, little nutrient at the termination of fermentation, and otherwise comply with a need to avoid commencement of the fermentation with a syrup containing more than about 20% dissolved carbohydrate.

Insofar as the inventor herein is aware, the existence of disadvantages in fermenting a completely saccharified starch have received relatively little attention from the art. Instead the art has concerned itself with improving liquefaction, e.g., U.S. Pat. No. 3,912,590, saccharification to pure glucose, e.g., U.S. Pat. No. 4,017,363, resolving processing difficulties, e.g., U.S. Pat. Nos. 3,922,196 to 3,922,201 and 4,009,074; and with avoiding high thermal energy requirements for starch liquefaction and saccharification, e.g., U.S. Pat. No. 4,092,434. Some of the above referenced U.S. Pat. Nos., particu-

larly 4,009,074 and 4,092,434, teach that ungelatinized starch, i.e., granular starch can be liquefied enzymatically at relatively low temperatures.

However, the inventor herein is not aware of any efforts by the art to integrate low temperature enzymatic liquefaction of starch with conduct of the fermentation so as to achieve improvements in the fermentation process, e.g., improved fermentation efficiency, and/or reduced fermentation time, and/or fermentation with a fermentation broth containing more than about 25% solids content. The last reduces water and energy costs.

The rationale of the present invention derives from a discovery that the fermentation may be carried out on a slurry of solid and completely ungelled starch, i.e., granular starch, dosed with alpha-amylase and glucoamylase. During the course of the fermentation the starch is enzymatically liquefied and saccharified into fermentable sugars and the sugars are fermented. Control over the fermentation rate is possible through variations in the starch concentration in the slurry, by preconditioning of the starch, and through variations in the concentration and proportions of alpha amylase and glucoamylase in the slurry.

## OBJECTS OF THE INVENTION

The principle object of this invention is to provide an improved fermentation process adapted for fermentation conversion of granular starch into ethanol.

One object of this invention is to provide a fermentation process with improved thermal efficiency.

Another object of this invention is to provide a rapid fermentation procedure.

An additional object of this invention is to provide a fermentation process that ferments a broth containing therein more than 25% by weight of substances convertible to ethanol.

Further objects and the advantages of this invention will become apparent from the description which follows.

## BRIEF STATEMENT OF THE INVENTION

Briefly stated, the present invention involves use of a fermentation medium which contains suspended granular starch particles, alpha-amylase, and a glucoamylase. The enzymes are relied upon to liquefy the solid starch particles and to saccharify the dissolved starch to the fermentables glucose and maltose during the course of the fermentation.

The proportions of granular starch in the medium and of each enzyme are set to provide a controlled release of fermentables to the yeast, allowing thereby control over the fermentation reactions. Characteristic of practice of the present invention is recovery of enzymes from the fermentation medium for use anew. Any undissolved starch particles remaining in the fermentation broth at the time the fermentation is halted contain thereon considerable amounts of alpha-amylase. Removal of residual starch particles from the fermentation broth for a later refermentation is a preferred way to recover enzymes.

According to one preferred embodiment of the invention, the enzymatic liquefaction, saccharification and fermentation circumstances are set to generate alcohol at near to a linear rate over the principal course of the fermentation.

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Optionally, the present process contemplates a pretreatment step in which a starch slurry is treated with an alpha-amylase and, optionally, a glucoamylase at temperatures below the initial gelatinization point of granular starch. (Which temperature is conventionally given as being not less than about 62° C. for cornstarch.) The pretreatment generates a small proportion of fermentables in the slurry so that the microorganism has nutrient immediately available for initiating fermentation.

#### GENERAL DISCUSSION OF THE INVENTION

For further understanding of this invention reference is now being made to the attached drawings wherein:

FIG. 1 is a graph showing pH v. activity for representative alpha-amylases at 37° C.

FIG. 2 is a graph showing disappearance of solid starch from starch slurried at 34° C. and in the presence of alpha-amylase over a period of time (Example 9, run 1).

FIG. 3 is a graph showing disappearance of solid starch under the conditions of the FIG. 2 graph when glucoamylase is also present (Example 9, run 4).

FIG. 4 is a graph showing the disappearance of starch under the conditions of the FIG. 2 graph when the slurry is fermented (Example 9, runs 2 and 3).

FIG. 5 is a graph showing the disappearance of solid starch under the conditions of the FIG. 3 graph when the slurry is fermented (Example 9, runs 5 and 6).

FIG. 6 is a graph showing the amylase and glucoamylase activity in the fermentation broth over the course of the fermentation illustrated by FIG. 5.

FIG. 7 is a flow sheet showing one mode of how fermentation of granular starch is integrated with an alcohol distillation; and

FIG. 8 is a flow sheet showing another mode of integrating the fermentation of granular starch with an alcohol distillation.

FIG. 9 is a graph showing the effect on fermentation results of different starch concentrations (Example 11).

Fermentation according to practice of this invention depends upon conducting all of the sequential reactions involved in converting granular starch into ethanol inside the same pot under the temperature and pH circumstances of 25°-38° C., pH 3-7 adapted to cultivation of the alcohol producing microorganisms such as saccaromyces. The reactions may be described as follows:

starch→solubilized dextrins→glucose and  
maltose→alcohol

As may be seen in FIG. 1, the activity of typical commercially available alpha-amylase enzymes on dissolved starch at pH 3.5 to 6, and 37° C. is substantial. The test methods were the Novo FA-Method, which is a modification of the classic Sandstedt, Kneen and Blish (SKB) analysis. However, results comparable to the curves shown in FIG. 1 will be obtained with any test method. Once the starch has been liquefied, the alpha-amylase contributes significantly to hydrolysis of the dextrins into fermentable sugars at fermentation pH and temperature conditions.

It has not been widely appreciated heretofore that alpha-amylase liquefies granular starch at a significant reaction rate. FIG. 2 shows both the cumulative disappearance of solid ungelled starch i.e., granular starch, over a period of time at 34° C. and, the corresponding increase in dissolved carbohydrate. It is noteworthy that (under the conditions tested, see Example 9 hereinafter) while the alpha-amylase dissolved only about 20%

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of the granular starch after 216 hours, when fermentation is carried out with a like slurry close to 40% of the granular starch had gone into solution after 216 hours. The fermentation results are illustrated in the graph of FIG. 4.

Inclusion of glucoamylase along with the alpha-amylase in the starch slurry produces more striking results. As can be seen from the test results illustrated in FIG. 3, (also Example 9 hereinafter) a combination of alpha-amylase and glucoamylase can dissolve about 60% of the granular starch in the slurry over 216 hours. Concurrently fermenting removed about 90% of the granular starch from the slurry, as is illustrated in FIG. 5.

The progression of from 20% to more than 90% disappearance of granular starch from a slurry under the cumulative influence of alpha-amylase, glucoamylase and fermentation demonstrates the feasibility of a single pot conversion of granular starch into ethanol. Moreover since enzyme concentration and proportions and starch concentration may be varied at will, a high degree of engineering flexibility exists for practice of this invention.

Conduct of the liquefaction hydrolysis and fermentation reactions concurrently inside the fermentor has numerous theoretical and practical points of superiority over conduct of the same reactions in three separate stages, the system heretofore employed to the greatest extent. On the whole, as may be noted from the results illustrated by FIGS. 1-4, concurrent conduct of the reactions accelerates the liquefaction and hydrolysis conversion reactions, thereby generating several points of superiority for the process of this invention over prior art practices.

Thus, liquefaction of starch to a (hot) dextrin solution has long faced both product loss and processing difficulties due to occurrence of starch retrogradation reactions in the hot dextrin. The concurrent liquefaction of the starch and saccharification of the dextrins which take place according to practice of this invention avoid conditions conducive to the starch reversion reactions.

Concurrent conduct of all the reactions at fermentation temperatures will, of course, save significant quantities of thermal energy, since the starch slurry need not be subjected to the elevated temperatures conventionally employed to liquefy starch. For instance, eliminating a conventional starch cooking process which cooks at 140° C. is estimated to save about 700 K cal per kg of ethanol product, which saving compares favorably to the about 7000 K cal per Kg combustion energy of ethanol.

A separate advantage is that any (non-dissolved) granular starch remaining at the expiration of the fermentation has not been degraded or even gelled (by a thermal treatment). Such starch may be recovered readily, then resuspended and cycled through the process as if it were fresh granular starch. Recycle of granular starch is contemplated for practice of this invention.

Conversion of dextrins into glucose when carried out by a microbial glucoamylase (AMG) on high solids content syrups is accompanied by an enzyme catalyzed reverse reaction wherein glucose polymerizes into polysaccharides such as isomaltose, a sugar which is non-metabolizable by the yeast. The reaction rate of the reversion reactions is some function of glucose concentration in the syrup. Therefore, a concurrent generation of glucose and fermentative removal of glucose from

the syrup should prevent even a small loss in fermentables due to glucose reversion reactions.

Fermentative conversion of the usual saccharified dextrin solution to alcohol often faces the yeast with more than enough carbohydrate nutrient i.e., of glucose and maltose, in the early stages of fermentation and a deficiency of carbohydrate nutrient in the late stages of fermentation. Practice of this invention avoids the excess of available carbohydrate nutrient during the early stages and, makes fermentable sugars available to the yeast during the late stages.

The ultimate fermentation broth can be expected to contain some of each of the individual materials in the above reaction sequence. It is noteworthy that the solid starch particles may be separated out easily (by centrifugation for example). The ethanol may be separated out easily (by distillation for example). The yeast can be counted upon to minimize the glucose content in the fermentation broth. Only the dextrans can neither be separated out readily, nor be converted by the yeast.

The dextrin content in the ultimate fermentation broth represents, at least potentially, a loss in the system. Accordingly, a preferred practice of this invention involves enzyme concentrations and proportions for an essentially complete rapid saccharification of the dextrans into fermentable sugars, so that dextrin content in the fermentation medium is maintained at a low level throughout the fermentation. Since the metabolic transformations carried on by the yeast insure that the glucose disappears quickly, the fermentation medium contains little glucose and dextrans at all times and over the course of the fermentation, considerable granular starch in ever decreasing amounts, and ethanol in ever increasing amounts.

#### ENZYME CONSIDERATIONS

Allusion has already been made as to how practice of this invention involves fermenting at the temperature and pH circumstances that are optimum for the ethanol producing microorganism, i.e., pH 3-7, 25°-38° C. Although the fermentation circumstances are not also optimum temperatures and pH for state-of-the-art commercially available alpha-amylases and glucoamylases, such enzymes are effective for practice of this invention in economically realistic enzyme concentrations. Actually, the pH conditions for fermentation do correspond closely to the optimum pH for commercially available saccharification enzymes, i.e., the glucoamylases. In the present process complete saccharification to glucose is favored by the gradual solubilization of granular starch which takes place. Presumably then, the enzyme always faces dextrin in low concentration. In addition, fermentation removal of the glucose throughout the fermentation maintains a low glucose content in the fermentation medium. The glucoamylase also faces glucose in low concentration. In consequence, the glucoamylase is used so effectively that economically feasible dosage levels of glucoamylase (AMG) can be employed for practice of this invention, namely a glucoamylase dosage of 0.05-10.0 AGU/g of starch preferably 0.2-2.0 AGU/g starch.

One AG unit (AGU) is the amount of enzyme which splits one micromol of maltose per minute at 25° C. and pH 4.3. A commercially available liquid form of glucoamylase (AMG NOVO 150) has an activity of 150 AGU per ml. (See Ford et al, Biochem. Vol. 54 (1973) 120.)

The dosages provided above for glucoamylase only approximate the effective concentration of the enzy-

matic saccharification activity in the fermentation broth. Some unknown proportion of the saccharification activity is contributed by the alpha-amylase. Commercially available alpha-amylases will produce significant amounts of sugars, such as glucose and maltose, as is evidence on FIGS. 2 and 4.

Indeed, addition of the alpha-amylase from *Aspergillus oryzae* (e.g., Fungamyl®) to wort has been suggested to the brewing industry. This particular enzyme saccharifies dextrans to maltotriose and maltose. Thus, although the purpose of the alpha-amylase is to liquefy the starch, its saccharification propensity also make the alpha-amylase some part of the saccharifying enzyme content.

On the other and, less than all of the glucoamylase dose added to the granular starch slurry may be active in catalyzing saccharification reactions in the solution. The glucoamylase proportions between starch solids and the liquid. A set of measurements carried during fermentation of a 20% by weight starch suspension indicated that about 70% of the glucoamylase is present in the liquid phase 6 hours after initiating fermentation, and about 85% is present after 72 hours. The results are illustrated in FIG. 6.

The results illustrated in FIG. 6 indicate, also that little, if any, loss of dissolved enzyme has occurred after 216 hours of fermentation. Accordingly, removal of the ethanol product from the fermentation broth by a recovery technique that does not subject the fermentation broth to temperatures that deactivate the glucoamylase enzyme, e.g., distillation under vacuum, allows recovery of the dissolved glucoamylase for use anew in fermentation of particulate starch.

A further point of interest to practice of this invention is that commercially available glucoamylases contain some alpha-amylase activity, and indeed, it is possible, but not practical, to ferment particulate starch in the presence solely of glucoamylase.

For practice of this invention, addition of alpha-amylase to the slurry of particulate starch is contemplated. With certainty all that can be said is that an effective amount of alpha-amylase is added. Aside from the uncertain amount of alpha-amylase activity contributed by the glucoamylase, the effective activity of the alpha-amylase may be quite different from the unit activity values given by the supplier. The activity of alpha-amylase is pH dependent, and as is illustrated by FIG. 1 for three diverse commercially available alpha-amylases, may be different at the pH range selected for the fermentation, than at the test conditions employed by the suppliers for their reported unit activity values. Trial and error tests can readily establish the most effective dosages for alpha-amylases not herein exemplified.

In any event the alpha-amylase dosage range for fungal alpha-amylases contemplated for practice of this invention is 0.02 FAU/g (Fungal Amylase Units) to 2.0 FAU/g of starch, preferably 0.05-0.6 FAU/g. One FAU is the amount of enzyme which breaks down 5260 mg of starch per hour under a standardized set of conditions. One FAU corresponds to approximately 25 SKB units, see Cereal Chemistry, Vol. 16 (1939) page 712-723. For Bacillus alpha-amylases the range is 0.01 KNU/g to 0.6 KNU/g, preferably 0.05 to 0.15 KNU/g, the NU (or Novo Unit) being the amount of enzyme which breaks down 5.26 mg of starch per hour under a standardized set of conditions. One KNU is 1000 NU.

The uncertainty as to the real activity of both the glucoamylase and the alpha-amylase in the fermenting

slurry will require some cut and try experimentation to achieve acceptably optimum operating conditions for any particular commercial installation constructed for practice of this invention. Some guidelines can be provided for optimization test efforts.

Increasing the alpha-amylase dosage with a constant glucoamylase content, increases the fermentation rate. Increasing the glucoamylase dosage with a constant alpha-amylase content increases the fermentation rate. Holding enzyme dosage constant, and increasing the starch content in the slurry increases the fermentation rate.

When all is said and done, the optimum alpha-amylase dosage may well exceed dosages heretofore recommended for liquefying starch; the optimum glucoamylase may well exceed dosages recommended for saccharifying syrups. However, enzyme dosage levels should not be confused with enzyme usage. Substantial proportions of the enzymes dosed into the starch slurry may be enzyme recovered from the fermentation broth for use anew to ferment granular starch.

A further consideration arising from employment of the enzymes at fermentation temperatures is that although the enzymes exhibit low relative activity, for example, activity of the alpha-amylase from *B. licheniformis* at fermentation temperatures is not more than about 25% of maximum activity, the low relative activity is counterbalanced by the extended duration of the 48-120 hours of fermentation, and by the extended half-life of enzymes that have not been subjected to elevated temperatures. As near as can be ascertained more than 90% of the enzymes activity remains after 72 hours of fermentation.

The alpha-amylase of *B. licheniformis* (e.g., Termamyl®), is sufficiently stable to withstand brief exposures to still pot temperatures. Thus, recycle of stillage can be used as a way to recycle alpha-amylase. In the main, however, recovery of enzyme in recycled stillage will require care to avoid subjecting the fermentation broth to ethanol stripping temperatures that deactivate the enzymes. The alcohol might, for example, be vacuum stripped from the fermentation broth and such stillage recycled to recover the enzymes.

A particularly advantageous way to recover enzymes for reuse, particularly the alpha-amylase is to conduct the fermentation so as to leave some granular starch in the fermentation broth when fermentation is halted. As is illustrated in FIG. 6, the alpha-amylase proportions itself between the granular starch solids and the solution with only about 25% of the alpha-amylase dosage to be found in the liquid (using the Phadebas® Amylase test, a variant of the method mentioned by Ceska et al Clin. Chim. Acta, Vol. 26 (1969) p. 437). Accordingly, recovering residual granular starch for renewed fermentation recycles a large proportion of the alpha-amylase, and a minor proportion of the glucoamylase.

It should, of course, be appreciated that the dosage ranges for both alpha-amylase and of glucoamylase described above are intended to be the total of recycled and freshly added enzymes.

#### PROCESS CONSIDERATIONS

As has already been pointed out, fermentation of a granular starch slurry has completely different characteristics than fermentation of a syrup. Generally about 20% solids in solution is considered the maximum sugar content in a fermentation medium with higher concentrations creating difficulties at the onset and at the end

of fermentation. No like limits exist on fermentation of a starch slurry. Concentration of starch in the slurry may vary from 10-45% with no discernable consequence at the onset of fermentation. Increasing starch concentration (at constant enzyme dosages) speeds up the fermentation rate, or conversely, allows for lowering the enzyme dosages required to achieve a given fermentation rate. In any event fermenting until the broth has 7-10% alcohol, as is prevalent in the fermentation arts, is still possible. The excess (residual) granular starch may be recovered, along with substantial amounts of enzymes and subjected to renewed fermentation. Thus, control over starch concentration is a major process parameter for practice of this invention.

One preferred mode of this invention is fermentation of a granular starch slurry having 25-40% starch by weight.

Fermenting a 25-40% starch slurry with common baker's yeast will invariably result in residual starch when fermentation has proceeded to the intended alcohol content levels e.g., 7-10% dependent on the micro-organism used and, therefore, recycle of the enzymes on the starch particles occurs when the residual starch is again fermented. However, even when a 10-25% starch slurry is fermented, preferred practice of this invention is to halt fermentation before complete disappearance of the granular starch, for fermentation anew. Recycling of starch is a facile way to recover enzymes for reuse.

According to one preferred mode of this invention, the (granular) starch and yeast are removed together, e.g., by centrifugation, and along with fresh granular starch and makeup enzymes become the fermentation charge.

Another difference between fermentation of granular starch according to practice of this invention and fermentation of a sugar syrup is that the granular starch slurry will normally be fermented at near to a linear rate during the main course of the fermentation. At the onset of fermentation a lag period may exist, while near the end of the fermentation the alcohol content may have built up to levels that interfere with the fermentation. In between, i.e., during the main course of the fermentation, the fermentative release of carbon dioxide and buildup of ethanol content proceed at near to a linear rate as is illustrated by FIGS. 4, 5 and 9. The linearity may be difficult to avoid in the starch slurry fermentation system of this invention, since fermenting slurries of different initial starch concentrations at any given enzyme dosage alters the fermentation rate, but does not alter the near to linearity at which CO<sub>2</sub> is generated. Changing enzyme proportions alters the fermentation rate without, it seems, altering the near to linear generation of CO<sub>2</sub> during the main fermentation.

It is believed that the near to linear rate of fermentation herein achieved is attributable to a near to linear rate at which the granular starch liquefies. The dextrins content in the fermentation broth is low (e.g., about 0.25%) during the main fermentation, as is the glucose content. The low measured dextrin and glucose levels leave liquefaction of granular starch as the limiting reaction, and, if linear, the reason for a linear fermentation rate. Conduct of fermentation at a near to linear rate as is a preferred practice of this invention is then keyed to having granular starch always present in the fermentation broth during the main fermentation.

The main fermentation can be considered to end at a level of alcohol content of about 7%, where common baker's yeast is unable to consume the maltose and glu-

cose nutrient as rapidly as the enzymes generate available nutrient through liquefaction and saccharification. Consequently, the fermentation rate (as measurable by CO<sub>2</sub> production) decreases and linearity is lost. The fermentation may be allowed to continue thereafter (at a non-linear rate) until the desired alcohol content in the fermentation broth is reached, and doing so is contemplated in practice of this invention.

Deviations from linearity in the fermentation rate as will typically occur at the end of the fermentation can cause buildup of carbohydrates in solution, both of fermentables and polysaccharides. Some engineering expedients (individually and in combination) contemplated for use in practice of this invention to avoid loss of the dissolved nutrients are:

(1) removing the starch (after the main fermentation) then continuing to ferment the starch free broth.

(2) vacuum stripping the fermentation broth to reduce alcohol content so that the microorganisms can function more effectively;

(3) recycling nutrient containing still bottoms for renewed fermentation.

To a large extent engineering trade-offs will be required in arriving at optimum process details, and the trade-offs may vary for each particular installation. For instance to achieve the most rapid fermentation reasonable, high starch content, high enzymes dosage are indicated, but then the consequential rapid fermentation tails off into generation of a level of nutrients in the fermentation broth that dictates recovery of the nutrients, or, alternatively that fermentation be halted at a relatively low alcohol content. If relatively low fermentation rates are acceptable, then (with high starch content slurries) enzyme dosage is relatively low and nutrient losses can be held to levels heretofore accepted by the fermentation arts. If maximum yield of alcohol is a principal objective, then low starch content slurries, moderate alpha-amylase dosage, high glucoamylase dosage might be employed.

In any event, preferred practice of this invention is to halt fermentation while granular starch yet remains in the fermentation broth. The granular starch residue is removed and fermented anew. Much of the alpha-amylase and a minor proportion of the glucoamylase are recovered thereby. Ordinarily, recovery of the starch will also remove from the fermentation broth much, or even all of the alcohol producing microorganisms. Starch and microorganism may be separated, if desired, but seeding a fermentation charge with recycled granular starch and microorganisms removed with the starch is desirable for practice of this invention.

Allusion has already been made as to how practice of this invention saves considerable thermal energy. However, just as the starch is never subjected to the thermal conditions used for liquefactions, the starch is not thermally sterilized. In all likelihood the granular starch will add contaminating microorganisms to the fermentation medium. Under the circumstances then, seeding the fermentation medium with the great number of the ethanol producing microorganism that might accompany recycled granular starch can be advantageous. Through their great numbers, the recycled microorganisms overwhelm any contaminating microorganisms, thereby dominating the fermentation, as is, of course, desired.

Allusion has been made to the brief lag phase that has been observed at the onset of fermentation in almost all instances. During the lag phase multiplication of the

microorganisms takes place and/or carbohydrate nutrients are generated from the granular starch. Pretreatment of the starch slurry with either or both enzymes for up to 20 hours at from 30° C. to 60° C. will serve to hasten the commencement of fermentative generation of ethanol in the fermentor. The enzymatic pretreatment serves to generate carbohydrate nutrient in the slurry before the microorganisms are introduced.

## EXEMPLARY PRACTICES OF THE INVENTION

One preferred mode of the present invention is illustrated in FIG. 7 wherein it can be seen that alpha-amylase, glucoamylase, water with essential nutrients therein, granular starch and an ethanol producing microorganism, e.g., brewer's yeast, are all added to batch fermentor 10. An essentially concurrent addition of all the ingredients is contemplated. Thereafter fermentation is carried out at usual temperature and pH conditions for ethanol production, e.g., at pH 5, 38° C. over a suitable period of time, e.g., 160 hours. Then the fermentation mixture is subjected to centrifugation in centrifuge 12, to separate yeast and any unconverted starch particles from the fermentation broth. The fermentation broth then passes by way of line 14 directly into still 16, wherein the alcohol content is stripped for removal overhead in line 18. The stillage is taken off as bottoms through line 20. Some of the stillage is recycled by way of heat exchanger 24 to become part of the feed water for the fermentation, and the balance of the stillage is discarded through line 22, as a purge stream.

The system illustrated by FIG. 7 is particularly adapted to the instances where a single pass complete conversion of the granular starch is desired, as would take place when a 10-20% starch slurry is fermented. The mode of FIG. 7 is particularly adapted also to use therein of thermally stable alpha-amylases, since such enzymes (e.g., Termamyl®) can withstand brief exposure to still pot temperatures. The enzymes notably the thermally stable alpha-amylase are returned to fermentor 10 in the water recycle.

The mode of FIG. 7 can be operated as a continuous fermentation, in which instance fermentor 10 might, for example, be modified so as to provide an elongated path for the fermenting slurry to travel (in plug flow) from inlet to outlet.

FIG. 8 illustrates a mode wherein fermentation is halted when unconverted granular starch yet remains in the fermentation broth. Fresh granular starch, enzymes and optionally yeast are charged into fermentor 110, along with recycled yeast and granular starch. The fermented broth passes to centrifugal separator 120 wherein yeast and unconverted granular starch are separated out for recycle by way of line 130 back to fermentor 110. Some of the yeast and starch is removed to a purge line 125. The centrifuged broth passes by way of line 140 into still 160 for separation into the alcohol overhead, removed through line 180, and still bottoms, removed by way of line 200. Some of the stillage is purged through line 220, while the balance is cooled in heat exchanger 240 before return to fermentor 110. The mode of FIG. 8 can, of course, be constructed for continuous operation.

Since as much as 75% of the alpha-amylase and about 15% of the glucoamylase values are recoverable in the starch recycle, the mode of FIG. 8 can be employed with a heat labile alpha-amylase. Complete thermal inactivation in still 160 of the alpha-amylase left in the centrifuged broth can be tolerated. Indeed, recycle of

stillage may be omitted altogether in practice of the mode of FIG. 8.

By and large, the mode of FIG. 8 is intended for fermenting high starch concentration slurries; 20-45% being the general range contemplated; 35-40% being the preferred range. Conveniently, the starch slurry from a wet corn milling operation can be fed directly into a fermentation system adapted to practice of this invention, e.g., the mode of FIG. 8. The solids content of a wet mill starch slurry is close to 40% starch by weight.

The quantities of yeast initially charged into the fermentation vat may be in accord with prior art practices for ethanol fermentation, and can vary widely since the yeast cells will multiply during the course of the fermentation. Recycle of yeast cells is not necessary. Removal of the yeast from the residual starch particles prior to recycling of the residual starch is contemplated. However, it is noted once again that practice of the present invention do not include inherently a thermal treatment of the starch i.e., thermal conditions that would heat sterilize the starch. It might well be advisable to charge relatively large proportions of yeast cells into the fermentation in order to help overcome the likelihood of (inadvertent) contamination. Antibiotics may be added to the fermentation medium to suppress growth of contaminating microorganisms, and cold sterilization techniques could, of course, be employed on the entering materials.

As has already been pointed out, practice of this invention controls the fermentation rate by releasing metabolizable sugars to the yeast at a controlled rate. This is different from what has been done heretofore. The objectives of prior art suggestions to treat solid starch with enzymes prior to fermentation and/or to include enzymes in the fermentation medium are to conserve energy and/or to improve fermentation efficiency, but are not suggestions to alter the character of the fermentation so as to achieve a near to linear fermentation rate. Certainly practice of the present invention conserves energy as compared to high temperature starch liquefaction. If anything, more thermal energy is conserved. The present process operates with high fermentation efficiency, in part because product losses due to starch retrogradation incomplete saccharification and incomplete fermentation of fermentables may be reduced. The ability to tailor the fermentation rate through control of starch concentration and enzymes content and proportions includes the capability of creating in the end a fermentation broth product with minimal carbohydrate content.

Thus, vis a vis prior art suggestions which superficially resemble some of the practices of this invention, the more comprehensive objectives of this invention are reflected into a great many detail features believed to be unique to practice of this invention, including notably enzyme recycle and starch recycle.

#### SPECIFIC EXAMPLES

For further understanding of this invention the following specific examples of practice thereof are posed.

##### EXAMPLE 1

A slurry containing 50 g granular corn starch (91.3% dry substance) in 150 g water, is adjusted to a Ca++ content of 7 mg/l (in final volume) and pH to pH 5. 65  $\mu$ l Termamyl® 60 L (alpha-amylase) and 135  $\mu$ l of Spiritamylase® 150 L (glucoamylase) are added. The

final weight is adjusted to 250 g by addition of water, and the suspension stirred on a water bath for 18 hours at 60° C.

After 18 hours, 150 ml of the slurry (approximately 162 gm) are transferred to a fermentation flask cooled to 30° C. and to the flask are added:

2 ml yeast extract solution (20 g DIFCO yeast extract in 100 ml of distilled water)

2 ml antibiotics (1.25 g Streptomycin, + 1.25 g penicillin in 150 ml distilled water)

5 ml yeast suspension (6 g bakers yeast/100 ml distilled water)

Spiritamylase-150 L (glucoamylase) as in Table 1.

The flask is equipped with a magnetic stirring rod, and fitted with a fermentation trap containing 98% sulphuric acid. After an initial weighing, fermentation is conducted at 30° C. for 6 days and the weight loss of CO<sub>2</sub> is measured by weighing the flask from time to time over the 6 days. The results are tabulated below:

TABLE 1

Run	AMG-150	CO <sub>2</sub> Weight Loss				RTS*
		18h	91h	115h	138h	
1	65 $\mu$ l	4.94g	14.22	14.36	14.52	3.35%
2	130 $\mu$ l	5.46	14.36	14.57	14.64	2.10%

\*Residual Total Sugar given in % of original starch feed, the amount of starch being converted to equivalent, weight of glucose

The analytical method used is the Minnesota method for the determination of total sugars (Whistler & Wolfrom: Methods in Carbohydrate Chemistry I, 1962 p. 388).

The measured RTS values cover both dextrans and granular starch as no filtration of the media is performed. (Note that in the following Table 8 B, the dextrin content in the fermentation is measured after filtering the fermentation broth, i.e., eliminating the contribution of the granular starch).

##### EXAMPLE 2

To a slurry of 30 g granular corn starch (91.3% dry substance) in 140 g water adjusted to 7 mg Ca++/l and pH 5 is added 30  $\mu$ l (0.1% on dry substance) Termamyl® 60L (alpha-amylase) then stirred at 60° C. for 18 hours.

After the 18 hours pre-treatment, the flask is cooled to between ambient and 30° C., then yeast extract, antibiotics and yeast are added as in example 1. After addition also of 50  $\mu$ l (0.165% on dry substance) of Spiritamylase® 150L (glucoamylase), the flask is fitted with a fermentation trap containing 98% sulphuric acid. After weighing, the flask is placed on a magnetic stirring device and fermented at 30° C. for 160 hours. The weight losses of CO<sub>2</sub> are measured by weighing from time to time. After 111 hours of fermentation approximately 50 grams of slurry is withdrawn and analyzed for total sugars content and for alcohol content (by cryostatics). (In further conduct of this run corrections are made for the withdrawn material.) At the end of the fermentation, at 160 hours, the analysis is repeated. The results are tabulated below.

TABLE 2

Run No.	Cumulative CO <sub>2</sub> losses in grams:				
	24h	87h	111h	136h	160h
1	5.78g	12.31	12.83	12.68	12.69
2	5.42g	11.85	12.57	12.47	12.48

Alcohol Content

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TABLE 2-continued

Run No.	RTS		% w/w	
	110h	160h	110h	160h
1	5.2%	4.3%	10.50%	10.77%
2	7.2%	6.4%	10.22%	10.55%

The fermentation was repeated with a split in glucoamylase addition, 25  $\mu$ l being added in the pretreatment and 25  $\mu$ l being added with the yeast. Except for a somewhat larger CO<sub>2</sub> production during the first 24 hours, and a greater RTS content in the fermentation broth, the alcohol yield and cumulative CO<sub>2</sub> production were similar to the results reported above in Table 2.

## EXAMPLE 3

The fermentations described in Example 2 were repeated substituting however, 30  $\mu$ l of BAN 120L (an alpha-amylase from *B. subtilis*) for the 30  $\mu$ l of Termamyl® (an alpha-amylase from *B. licheniformis*).

The results are tabulated below.

TABLE 3A

Cumulative CO <sub>2</sub> losses				RTS		Alcohol Content % w/w	
24h	87h	111h	160h	110h	160h	110h	160h
5.24	12.13	12.80	13.21	5.7%	3.54%	10.28%	10.54%

The results obtained when addition of the glucoamylase is split between the pretreatment and the fermentation are tabulated below.

TABLE 3B

Cumulative CO <sub>2</sub> losses				RTS		Alcohol Content % w/w	
24h	87h	111h	160h	110h	160h	110h	160h
5.75	11.28	12.14	12.55	9.1%	7.03%	10.17%	10.45%

## EXAMPLE 4

A comparative test was carried out comparing conduct of a fermentation with liquefied starch and with solid starch according to the present process.

## A. Liquefied Starch

30 g of corn starch (91% dry solids content) slurried in 140 g of water, pH adjusted to pH 5 and 7 mg Ca++ added was dosed with 30  $\mu$ l of Termamyl® 60L, then the slurry was heated in a stirred laboratory autoclave at 2° C./minute to 100° C. and held at 100° C. for 30 minutes. The sample was weighed and the water loss (through evaporation) was replaced. The solution was cooled to 30° C. then dosed and fermented as in Example 1.

## B. Solid Starch

A like 30 g starch of slurry with 30  $\mu$ l Termamyl® was stirred for 21 hours at 60° C. on a water bath, then cooled to 30° C., dosed and fermented as in Example 1.

The results are tabulated below.

TABLE 4

Run No.	Procedure	CO <sub>2</sub> Losses After			
		19h	45h	114h	138h
1	B	4.24	8.37	12.69	13.03
2	B	4.05	8.27	12.60	12.90
3	B	4.10	8.19	12.69	13.09
4	A	3.83	8.72	11.66	11.65

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TABLE 4-continued

Run No.	Procedure	CO <sub>2</sub> Losses After			
		19h	45h	114h	138h
5	A	3.28	6.92	11.30	11.31
6	A	3.08	8.22	12.96	13.08

A high residual sugar content in the fermentation broth in runs 4 and 5 (procedure A) is believed to indicate that a significant level of starch retrogradation occurred.

## EXAMPLE 5

The pretreatment and fermentation of Example 2 was repeated, except that the 60° C. pretreatment was carried out at pH 5 and at pH 6 to ascertain whether the optimum pH activity pattern of the enzyme was material in starch treatment at below the temperature of starch gelation. The results tabulated below indicate that treatment at the pH optimum for the enzyme is nominally superior.

TABLE 5

Run No.	Initial pH	Cumulative CO <sub>2</sub> losses				RTS in %
		17h	41h	70h	166h	
1	5	3.90	7.97	10.38	12.3	5.3%
2	5	3.84	7.78	10.33	12.3	4.5%
3	6	3.88	7.50	10.00	12.11	5.7%
4	6	3.91	7.79	10.34	12.15	6.3%

## EXAMPLE 6

A. The pretreatment and fermentation of Example 2 was repeated with the 60° C. pretreatment time varied between 3 hours and 20 hours.

The results as tabulated below indicate surprisingly little differences between a 3 hour pretreatment and a 20 hour pretreatment.

TABLE 6A

Run No.	$\gamma$ -amylase Treatment at 60° C., hrs.	Weight losses as CO <sub>2</sub>				RTS content after 166 hrs. % of starch
		17h	41h	70h	166h	
1	20	3.90	7.97	10.38	12.30	5.3%
2	20	3.78	7.73	10.18	12.24	5.4%
3	6	3.71	7.33	10.30	12.07	4.5%
4	6	3.76	7.17	9.65	11.85	7.9%
5	3	3.76	7.28	9.99	11.62	9.3%
6	3	3.69	7.32	9.97	10.48	3.4%

B. The pretreatment was omitted entirely, with the yeast, granular starch and enzymes all mixed directly at 30° C. into the fermentation medium. The results are tabulated below.

TABLE 6B

Run No.	Weight losses as CO <sub>2</sub>				RTS content after 166 hrs. % of starch
	17h	41h	70h	166h	
1	1.25	3.75	6.56	11.52	7.0%
2	1.32	3.82	6.64	11.79	5.5%

## EXAMPLE 7

This example illustrates the use of different alpha-amylases at varying dosage levels. The pretreatment quantities and conditions, except as tabulated below, were those described in Example 2.

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TABLE 7A

Run No.	$\gamma$ -amylase dosage in % of dry matter		pH	Temp.
	Termamyl® 60L	Fungamyl® 800L		
1	0.1%, 30 $\mu$ l		5	60° C.
2		0.1%, 30 $\mu$ l	5	60° C.

As in Example 2, the samples are submitted to fermentation at 30° C. after addition of glucoamylase yeast, yeast extract and antibiotics.

Weight loss, glucose in solution, total sugars in solution (dextrins and glucose) are analyzed during the fermentation.

TABLE 8B

WEIGHT LOSSES AND SUGAR CONTENTS DURING FERMENTATION								
DEXTRINS AND GLUCOSE IN AQUEOUS PHASE								
RUN NO.	WEIGHT LOSSES AS CO <sub>2</sub>				SUGAR ANALYSES 69h		SUGAR ANALYSES 138h	
	16h	43h	69h	138h	GLUCOSE %	TOTAL SUGAR	GLUCOSE %	TOTAL SUGAR
1	1.24g	3.30	4.76	7.60	0.006%	0.11%	0.010%	0.14%
2	1.73g	3.81	5.30	8.36	0.004%	0.10%	0.009%	0.15%
3	2.38	5.17	7.18	10.40	0.008%	0.15%	0.004%	0.14%
4	2.34	4.23	5.84	8.25	0.009%	0.22%	1.76%	2.07%
5	3.41	6.61	8.77	10.69	0.24%	0.49%	3.87%	4.07%
6	0.49	1.93	3.44	7.48	0.005%	0.10%	0.007%	0.14%
7	0.88	2.97	5.35	9.81	0.005%	0.10%	<0.001%	0.15%
8	1.29	4.31	6.77	10.91	0.009%	0.17%	1.88%	2.07%
9	2.54	7.69	9.88	10.35	2.48%	2.72%	>8.17%	8.34%
10	2.42	4.90	6.87	10.08	0.017%	0.15%	0.002%	0.14%
11	1.46	3.46	5.04	8.05	0.007%	0.13%	<0.001%	0.13%

3	0.1%	6	60° C.
4	0.05%	6	60° C.
5		5	55° C.
6	0.02%	5	55° C.
	0.01%		

As in Example 2, the  $\alpha$ -amylase treated samples were submitted to fermentation at 30° C. after addition of glucoamylase, yeast, yeast extract and antibiotics. The results are tabulated below:

TABLE 7B

Run No.	Weight losses as CO <sub>2</sub>				RTS content after 166 hrs. % of starch
	17h	41h	70h	166h	
1	3.82	7.94	10.56	12.41	7.1%
2	3.57	7.36	10.19	12.37	6.6%
3	3.81	7.94	10.84	11.43	
4	3.64	7.34	10.11	12.25	7.3%
5	—	—	8.15	11.98	4.5%
6	2.38	5.57	8.64	12.46	6.2%

## EXAMPLE 8

This example illustrates the use of high starch content in the fermentation, with and without pretreatment.

The pretreatment quantities and conditions, except as tabulated below, were those described in Example 2.

The amount of glucoamylase added in connection with the fermentation is also shown below.

TABLE 8A

Run No.	Pretreatment at 55° C.	% Starch	Fungamyl® 800L	Glucoamylase AMG-150L in fermentation
1	None	20%	10 $\mu$ l	50 $\mu$ l
2	None	20%	20 $\mu$ l	50 $\mu$ l
3	None	20%	20 $\mu$ l	100 $\mu$ l
4	None	40%	20 $\mu$ l	50 $\mu$ l
5	None	40%	40 $\mu$ l	100 $\mu$ l
6	None	20%	None	50 $\mu$ l
7	None	20%	None	100 $\mu$ l
8	None	40%	None	100 $\mu$ l
9	None	40%	None	200 $\mu$ l
10	4 1/2 h	20%	None	100 $\mu$ l*
11	4 1/2 h	20%	20 $\mu$ l	50 $\mu$ l*

\*Glucoamylase added in connection with pretreatment.

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As in Example 2, the samples are submitted to fermentation at 30° C. after addition of glucoamylase yeast, yeast extract and antibiotics.

Weight loss, glucose in solution, total sugars in solution (dextrins and glucose) are analyzed during the fermentation.

TABLE 8B

WEIGHT LOSSES AND SUGAR CONTENTS DURING FERMENTATION								
DEXTRINS AND GLUCOSE IN AQUEOUS PHASE								
RUN NO.	WEIGHT LOSSES AS CO <sub>2</sub>				SUGAR ANALYSES 69h		SUGAR ANALYSES 138h	
	16h	43h	69h	138h	GLUCOSE %	TOTAL SUGAR	GLUCOSE %	TOTAL SUGAR
1	1.24g	3.30	4.76	7.60	0.006%	0.11%	0.010%	0.14%
2	1.73g	3.81	5.30	8.36	0.004%	0.10%	0.009%	0.15%
3	2.38	5.17	7.18	10.40	0.008%	0.15%	0.004%	0.14%
4	2.34	4.23	5.84	8.25	0.009%	0.22%	1.76%	2.07%
5	3.41	6.61	8.77	10.69	0.24%	0.49%	3.87%	4.07%
6	0.49	1.93	3.44	7.48	0.005%	0.10%	0.007%	0.14%
7	0.88	2.97	5.35	9.81	0.005%	0.10%	<0.001%	0.15%
8	1.29	4.31	6.77	10.91	0.009%	0.17%	1.88%	2.07%
9	2.54	7.69	9.88	10.35	2.48%	2.72%	>8.17%	8.34%
10	2.42	4.90	6.87	10.08	0.017%	0.15%	0.002%	0.14%
11	1.46	3.46	5.04	8.05	0.007%	0.13%	<0.001%	0.13%

3	0.1%	6	60° C.
4	0.05%	6	60° C.
5		5	55° C.
6	0.02%	5	55° C.
	0.01%		

As in Example 2, the  $\alpha$ -amylase treated samples were submitted to fermentation at 30° C. after addition of glucoamylase, yeast, yeast extract and antibiotics. The results are tabulated below:

TABLE 7B

Run No.	Weight losses as CO <sub>2</sub>				RTS content after 166 hrs. % of starch
	17h	41h	70h	166h	
1	3.82	7.94	10.56	12.41	7.1%
2	3.57	7.36	10.19	12.37	6.6%
3	3.81	7.94	10.84	11.43	
4	3.64	7.34	10.11	12.25	7.3%
5	—	—	8.15	11.98	4.5%
6	2.38	5.57	8.64	12.46	6.2%

## EXAMPLE 8

This example illustrates the use of high starch content in the fermentation, with and without pretreatment.

The pretreatment quantities and conditions, except as tabulated below, were those described in Example 2.

The amount of glucoamylase added in connection with the fermentation is also shown below.

TABLE 8A

Run No.	Pretreatment at 55° C.	% Starch	Fungamyl® 800L	Glucoamylase AMG-150L in fermentation
1	None	20%	10 $\mu$ l	50 $\mu$ l
2	None	20%	20 $\mu$ l	50 $\mu$ l
3	None	20%	20 $\mu$ l	100 $\mu$ l
4	None	40%	20 $\mu$ l	50 $\mu$ l
5	None	40%	40 $\mu$ l	100 $\mu$ l
6	None	20%	None	50 $\mu$ l
7	None	20%	None	100 $\mu$ l
8	None	40%	None	100 $\mu$ l
9	None	40%	None	200 $\mu$ l
10	4 1/2 h	20%	None	100 $\mu$ l*
11	4 1/2 h	20%	20 $\mu$ l	50 $\mu$ l*

\*Glucoamylase added in connection with pretreatment.

## EXAMPLE 9

This example illustrates the combined effect of the amylases and the yeast compared to the effect of amylase alone without fermentation.

No pretreatment of the starch with amylases. Both the hydrolysis and fermentation are run at 34° C. The starch content is 20% in this experiment, but 90 g are used (portions tripled compared to Example 2). In order to get quick fermentations, the yeast content is five times the content in Example 2; initial pH is 4.5

TABLE 9

EXPERIMENTAL CONDITIONS				
Run No.	$\gamma$ -amylase Fungamyl® 500 L	Glucoamylase AMG	Kind of Treatment	Analyzed for
1	120 $\mu$ l	None	Saccharification	Refractometric dry Subst.
2	120 $\mu$ l	None	Sacch. + Ferment	CO <sub>2</sub> losses
3	120 $\mu$ l	None	Sacch. + Ferment	$\gamma$ -amylase activity, alcohol cont.
4	120 $\mu$ l	300 $\mu$ l	Saccharification	as (1)

TABLE 9-continued

EXPERIMENTAL CONDITIONS				
Run No.	$\gamma$ -amylase Fungamyl® 500 L	Glucosylase AMG	Kind of Treatment	Analyzed for
5	120 $\mu$ l	300 $\mu$ l	Sacch. + Ferment	as (2)
6	120 $\mu$ l	300 $\mu$ l	Sacch. + Ferment	as (3) + glucosylase activity

The results of the analyses for the different runs are shown in the drawings as FIGS. 2-6. The amylase content in the liquid for runs 2 and 3 were about the same as is illustrated for runs 5,6.

The alcohol content in the fermentations has been analyzed (%w/w) and is tabulated below.

FERMENTATION TIME					
Run	24 h	48 h	72 h	216 h	216 h
3	1.19%	1.65%	2.59%	3.60%	3.66% (run 2)
6	2.9%	5.43%	7.40%	8.28%	8.32% (run 5)

## EXAMPLE 10

This example illustrates the possibility of submitting unreacted granular starch from a fermentation to a new fermentation after separation of starch and yeast from

The above results can be explained as follows:

Initially, 60 g of starch were present (90 percent dry matter) corresponding to 54 g of starch d.s.b. The starch consumed in the first fermentation is 22.75 g (glucose)  $\times 0.9 = 20.5$  g. This leaves about 33.5 g dry starch in the 140 g left in the fermentation broth after generation and release of about 10 g of CO<sub>2</sub>. The water-alcohol in liquid phase in the mixture will be about 110 ml, thus leaving about 40 g of water phase with an alcohol content of about 10 percent after the decantation removal of about 70 g of aqueous phase. About 50 g of water was added for the water makeup to the (second) fermentation.

Since 10 percent alcohol is near the toxic limits to the fermentation, a CO<sub>2</sub> loss in the second fermentation of about half of the CO<sub>2</sub> loss in the first fermentation is all that could be expected, and in fact, no more was obtained during fermentation for 45 hours. Over the first 24 hours of fermentation both fermentations generated CO<sub>2</sub> at about the same rate, indicating thereby comparable enzyme activity levels in the fermenting slurries.

## EXAMPLE 11

The purpose of this experiment is to show the influence of dry matter content of the granular starch slurry on the fermentation rate.

Each flask contains 150 g total weight. Yeast and yeast extract as in Example 9.

Run No.	Starch Content	Enzyme Dosage		Weight Losses as CO <sub>2</sub>					% Starch Conv. 167 h
		Fungamyl® 800 L	AMG 150 L	24 h	48 h	71 h	95 h	167 h	
1	10%	10 $\mu$ l	25 $\mu$ l	0.92	1.94	—	3.94	—	70%
2	20%	20 $\mu$ l	50 $\mu$ l	2.23	3.66	5.04	6.24	9.48	78.5%
3	25%	25 $\mu$ l	62.5 $\mu$ l	2.95	5.34	7.38	9.08	12.63	81.1%
4	30%	30 $\mu$ l	75 $\mu$ l	3.30	5.74	7.93	9.82	12.40	66.7%
5	40%	40 $\mu$ l	100 $\mu$ l	3.72	6.52	8.67	9.48	9.50	57.2%

the aqueous phase.

A 40% starch slurry (150 g final weight), pH 4.5, with 40  $\mu$ l Fungamyl® 800 L, 100  $\mu$ l AMG 150 L, yeast and yeast extract as in Example 9, is fermented at 30° C.

After 48 h, the unreacted starch and yeast is allowed to sediment. The aqueous phase is decanted off. To each fermentation flask an amount of starch is added of about 2 $\times$  the CO<sub>2</sub> losses (this brings the starch slurry concentration back to about its original value).

10  $\mu$ l Fungamyl® are added (to make up for the 25% alpha-amylase disappearing with the aqueous phase) and 100  $\mu$ l AMG 150 L are added (as the glucosylase is mainly lost with the aqueous phase).

The flasks are made up to 150 g with water. The fermentation is continued without addition of new yeast (the yeast sediments with the granular starch). The fermentation is followed by CO<sub>2</sub> weighing analyses.

Run	First Fermentation			Second Fermentation		
	CO <sub>2</sub> losses 24 h	Alc. % w/w 48 h	Alc. % w/w 48 h	Starch Added	CO <sub>2</sub> losses 21 h	CO <sub>2</sub> losses 45 h
1	3.62 g	8.29	8.60%	16.6 g	2.19 g	5.59
2	4.78 g	10.02	10.21%	20.0 g	4.60 g	5.38
3	5.49 g	10.61	10.37%	21.2 g	4.62 g	4.83
4	4.88 g	10.09	9.94%	20.2 g	4.65 g	5.46
5	3.83 g	8.93	8.85%	17.9 g	4.32 g	4.80

To avoid possible confusion about the details of practice of this invention, the carbohydrate nutrient has always been posed hereinabove as granular starch. Such reference only to granular starch is not intended as reference solely to starch in the separated out relatively pure forms (e.g., cornstarch, potato starch) widely available in commerce. The other forms of starch heretofore employed in fermentation practice, including, for example, corn grits, degerminated grains, cracked grains, even whole grains are directly fermentable according to the above described practice of this invention, and the term granular starch as employed in the claims hereinafter is intended to include within its scope, all starch in all forms.

I claim:

1. A fermentation process which consists essentially of fermenting a 10%-45% w/w aqueous slurry of granular starch for the production of ethanol with an ethanol producing microorganism in the presence of alpha-amylase and glucosylase, the conduct of said fermentation being characterized by low levels of dextrin and fermentable sugars in solution in the fermentation broth throughout the fermentation, and, thereafter recovering enzymes from the fermentation broth for use anew in fermentation of granular starch.

2. The fermentation process of claim 1 wherein the process further comprises halting the fermentation prior to complete disappearance of granular starch, and thereafter recovering residual granular starch from the

fermentation broth for later fermentation, enzymes being recovered on the residual starch.

3. The fermentation process of claim 1 carried out with a near to linear generation of CO<sub>2</sub> during the main course of the fermentation.

4. The fermentation process of claim 1 wherein the starch content of the slurry is in the range of 25-45% w/w and the granular starch and alcohol producing microorganism are removed from the fermentation broth for later fermentation, the enzymes being recycled with the granular starch.

5. The fermentation process of claim 1 wherein a thermostable alpha-amylase is employed and de-alcoholized fermentation broth is used anew in fermentation of granular starch, the de-alcoholized fermentation broth containing therein active alpha-amylase.

6. The fermentation process of claim 1 wherein the granular starch is pretreated with at least one of said enzymes at temperatures not exceeding the initial gelatinization temperature of starch prior to the fermentation.

7. A fermentation process which consists essentially of fermenting a 10%-45% w/w aqueous slurry of granular starch for the production of ethanol with an ethanol producing microorganism in the presence of alpha-

amylase and gluco-amylase, the conduct of said fermentation being characterized by low levels of dextrin and fermentable sugars in solution in the fermentation broth throughout the fermentation, and a near to linear rate of CO<sub>2</sub> generation during the main course of the fermentation.

8. A fermentation process which consists essentially of fermenting a 10%-45% w/w aqueous slurry of granular starch for the production of ethanol at a temperature in the range of 25°-38° C. with an ethanol producing microorganism in the presence of fungal alpha-amylase in amounts within the range of 0.02-2.0 FAU/g of starch in the slurry or *Bacillus* amylase in the amount of 0.01-0.6 KNU/g of starch in the slurry and gluco-amylase in amounts within the range of 0.05-10.0 AGU/g of starch in the slurry, the conduct of said fermentation being characterized by low levels of dextrin and fermentable sugars in solution in the fermentation broth throughout the fermentation, and generation of CO<sub>2</sub> at a near to linear rate during the main course of the fermentation, and, thereafter recovering enzymes from the fermentation broth for use anew in fermentation of granular starch.

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